MECHANISMS OF RESISTANCE OF *PSEUDOMONAS AERUGINOSA* TO THE FOUR-QUINOLONES.

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ABSTRACT.

Mechanisms of resistance of *Pseudomonas aeruginosa* to the 4-quinolones were investigated by examining the outer membrane proteins and DNA gyrases of resistant clinical isolates. Minimum inhibitory concentrations (MICs) were determined for fluoroquinolones, imipenem, β-lactams, gentamicin, kanamycin, carbenicillin and tetracycline. Ciprofloxacin was the most active 4-quinolone, followed by ofloxacin and norfloxacin. Resistant clinical isolates had an MIC of ciprofloxacin of 4mg/l or greater, and resistance was shown to be stable after 20 passages on nutrient agar in all but one of the isolates. In several isolates cross-resistance with imipenem and β-lactams was seen. All clinical isolates were resistant to tetracycline, carbenicillin and kanamycin.

Outer membrane profiles of sensitive and resistant strains of *P.aeruginosa* were examined and shown to be variable. In highly resistant strains, resistance to 4-quinolones could not be attributed to a particular membrane alteration as the isolates were not paired. The predominant membrane alterations observed, however, were in proteins of 51kD, 36kD and 32kD. Low level quinolone resistance in a series of paired isolates was linked with an increase in expression of a 46kD protein and the decrease in expression of a 51kD protein, and ceftazidime resistance with the appearance of a 42kD protein. These proteins were found to be non-covalently associated with peptidoglycan suggesting that they act as porins. Experiments with EDTA also demonstrated that all of the quinolone-resistant strains carried OMP mutations.

DNA gyrases from 23 clinical strains were isolated from a novobiocin/sepharose column and assayed by gel electrophoresis. The level where quinolones inhibited 50% of gyrase supercoiling activity on relaxed pBR322 (IC_{50}), was determined for ciprofloxacin, ofloxacin, norfloxacin and novobiocin in all strains. In general the IC_{50}

concentrations were equivalent to, or greater than, the MIC of each drug. All IC_{50} values for clinical isolates were significantly greater than those of the reference strain PAO1, indicating that the strains possessed *gyrA* mutations. The clinical strains fell into three categories; those with a relatively low IC_{50} of 4-20 mg/l ciprofloxacin, those with an intermediate value of 50-100 mg/l ciprofloxacin and those with values greater than 150 mg/l ciprofloxacin. Five strains exhibited raised novobiocin IC_{50}s suggesting that they contained *gyrB* mutations. No correlation between MIC and IC_{50} values were observed.

GyrA mutations were analysed indirectly in 18 ciprofloxacin-resistant unpaired strains by restricting PCR fragments with the enzyme *SstII* which cleaves the fragment between amino acids 83 and 84 in the sensitive strain PAO1. *SstII* cleavage did not occur in 8 clinical isolates indicating that they possessed a *gyrA* mutation within this region. DNA gyrases were also investigated for mutations with pNJR3-2, a plasmid which carries a wild type *Escherichia coli gyrA* gene and confers quinolone sensitivity. Experiments to introduce the plasmid into *P.aeruginosa* by conjugation and electroporation were unsuccessful. Attempts to sequence the *gyrA* genes of selected clinical isolates were also unsuccessful, as transconjugants carrying the 300 base pair fragment were not selected.

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This thesis is dedicated to my grandmother, without whose encouragement I never would have started.

DECLARATION.

The research described in this thesis is the sole work of the undersigned author unless otherwise stated.

CONTENTS.

PRESENTATIONS.

Quibell,K., Sato,K., Osada,Y. and Amyes,S.G.B. (1993). DNA gyrases in 4-quinolone-resistant *Pseudomonas aeruginosa.* (Poster). *6th European Congress of Clinical Microbiology and Infectious Diseases.* Seville, Spain. March 28-31 1993.

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INTRODUCTION.

1.1 *PSEUDOMONAS AERUGINOSA.*

Pseudomonas aeruginosa was originally observed by medical practitioners as a blue-green stain that frequently appeared upon surgical dressings, and the nature of this coloured substance was first investigated by Fordos (1860). Later, in 1882, Gessard proved that the pigment was produced by a bacillus, named *Bacillus pyocyaneus*, which was easily isolated in pure culture. Attempts to determine the nature of the pigment produced by *B.pyocyaneus* lead to considerable confusion amongst investigators. Thumm (1895) claimed that a single pigment was produced and that this was identical to the blue-green pigment produced by common water bacteria, whereas Charrin and de Nittis (1898) claimed that the same strain of *B.pyocyaneus* was able to produce as many as four distinct pigments.

These findings were clarified by Jordan (1899) who separated *B.pyocyaneus* into four varieties; 1) pyocyanigenic and fluorescigenic, 2) pyocyanigenic only, 3) fluorescigenic only and 4) nonchromogenic. Jordan (1899) suggested that the chief difference between *Bacillus fluorescens liquefaciens*, later termed *Pseudomonas fluorescens*, and his fluorescigenic non-pyocyanin-variety of *B.pyocyaneus*, later termed *Pseudomonas aeruginosa*, was one of temperature, and perhaps their behaviour in animals. Jordan (1899) also mentioned that *P.fluorescens* might be a modified form of *P.aeruginosa*, and as such they were related.

The inability of investigators to distinguish between *Pseudomonas* groups persisted until 1943 when Seleen and Stark separated 199 cultures on their ability to grow at 5°C and 42°C, their action on milk, liquefaction of gelatin and reduction of nitrates. Sub-groups were also identified by pyocyanin production and the ability to utilise sucrose, acetic acid, lactic acid or tartaric acid as the sole carbon source. More recently the family *Pseudomonadaceae* has been classified by nucleic acid homologies, and *Pseudomonas* has been assigned five sub-groups on the basis of ribosomal RNA/DNA homologies (Palleroni *et al* 1973; Palleroni 1986).

P.aeruginosa was found to be widely distributed in nature, where it existed as a saprophyte, occasionally giving rise to pathological lesions and generalised infection in man (Lartigau 1898; Fraenkel 1917). *P.aeruginosa* was also one of the first microorganisms known to produce substances antagonistic to other bacteria. Bouchard (1889) observed that injecting small quantities of a culture of *P.aeruginosa* into rabbits previously injected with a virulent strain of *Bacillus anthracis* resulted in the rabbits' survival. Other investigators observed that old cultures and culture filtrates of *P.aeruginosa* were bactericidal for many microorganisms, and termed the substance they found pyocyanase (Emmerich and Low 1899).

P.aeruginosa is able to colonise humans, where it acts as a saprophyte, and is frequently present in small numbers in normal intestinal flora and the skin. *P.aeruginosa* is an opportunistic pathogen, presenting no problem to healthy individuals, but causing grave problems in patients with impaired resistance (Clarke 1989). It is a dangerous hazard in hospitals as it survives in many reservoirs, such as hospital humidifiers, weak disinfectants, brushes, mops and sinks (Lowbury 1975).

P.aeruginosa is only pathogenic in areas devoid of normal body defences; when mucous membranes or skin are disrupted by tissue damage, when intravenous or urinary catheters are used, or when neutropenia is present, for example during cancer therapy (Clarke 1989). *P.aeruginosa* is associated with a variety of clinical manifestations including infections of burns and surface wounds; urinary tract, ear and eye infections; infection of the cerebrospinal fluid in meningitis and colonisation of the lungs in cystic fibrosis (Clarke 1989). In the last decade *P.aeruginosa* has become the major cause of morbidity and mortality in patients with cystic fibrosis (Govan 1988). In many cases *P.aeruginosa* causes chronic disease.

P.aeruginosa colonises patients by attaching to mucous membranes, or skin, invading locally and then causing systemic disease (Pollack 1984). The process is promoted by pili and the production of various enzymes, toxins and pigments: exotoxin A and exo-enzyme S are protein synthesis inhibitors and elastase and alkaline protein are extracellular proteases. Virulence factors are also produced by *P.aeruginosa* and include haemolysin and phospholipase A, which break down lipids and lecithin (Liu 1979). Another characteristic of *P.aeruginosa* is that it produces pigments such as pyocyanin, a phenazine pigment which is a secondary metabolite produced during the biosynthetic pathway for aromatic amino acids. Pyocyanin is a strong iron chelating compound which enables the bacteria to grow in niches with low iron content, such as the lung environment in cystic fibrosis patients, and makes the organism highly adaptable (Clarke 1989).

P.aeruginosa provides a major obstacle to antimicrobial chemotherapy as it is highly resistant to many of the antimicrobial agents in use today (Nikaido and Hancock 1986). This resistance is brought about by three mechanisms; intrinsic resistance, adaptive resistance and the acquisition of new genetic material in the form of plasmids and transposons.

Intrinsic resistance is brought about by the impermeability of the cell wall of *P. aeruginosa.* The major porin protein in the cell wall of *Pseudomonas,* through which antibiotics can diffuse, is protein F and in isolates of *P.aeruginosa* the majority of F protein channels are either maintained in a closed state, or have very small diameters which block the passage of many antibiotics (Nicas and Hancock 1983). This, coupled with the fact that the lipopolysaccharide (LPS) on the pseudomonad cell wall is closely packed, thus protecting the cell surface, renders the bacterium impermeable to many hydrophobic and hydrophilic antibiotics (Nikaido and Hancock 1986). Another form of intrinsic resistance is chromosomally mediated, and in the case of the older β-lactam drugs this is brought about by an inducible chromosomally specified β-lactamase (Sabath and Abraham 1964). *P.aeruginosa* is also intrinsically resistant to kanamycin, brought about by a chromosomally encoded aminoglycoside phosphorylating enzyme. (Clarke 1989).

Adaptive resistance occurs when selective pressures affect bacteria, a classic case being lung colonisation of *P.aeruginosa* in cystic fibrosis patients. Here the lungs are colonised by wild type *P.aeruginosa,* and the high salt and low nutrient conditions there trigger *P.aeruginosa* into producing alginate, a highly viscous polymer which protects the bacteria by rendering them resistant to antibiotics and phagocytosis (Berry *et al* 1989). In cystic fibrosis this adaptive type of resistance is responsible for a persistent and recurrent infection, and is unstable in the absence of the selective pressure; in other situations adaptive resistance has been shown to be stable.

Plasmid-mediated resistance acquired by *P.aeruginosa* has major effects therapeutically. Plasmids may carry alternative genes which add to the intrinsic resistance of *P.aeruginosa.* The most common plasmids in *P.aeruginosa* are very large, have a narrow host range and belong to the P-2 incompatibility group (Jacoby 1986). *P.aeruginosa,* however, is also a host for a wide range of plasmids, and resistance genes carrying β-lactamases, aminoglycoside modifying enzymes, and resistance to tetracycline, sulphonamides and chloramphenicol, have been found to transfer into the bacteria (Clarke 1989). *Klebsiella* species have also been known to transfer resistance genes into *P.aeruginosa.*

The intrinsic resistance that *P.aeruginosa* exhibits to many antimicrobial agents was thought to have been overcome by the advent of the synthetic quinolone antibacterials as no natural antimicrobial agents resembled them. Within three months of the introduction of the modern fluoroquinolones, however, clinical reports of resistance in *P.aeruginosa* to ciprofloxacin were documented (Crook *et al* 1985; Roberts *et al* 1985). This highlighted the adaptability of *P.aeruginosa* to antimicrobial agents, and has lead to a worrying situation therapeutically as overall antimicrobial resistance in *P.aeruginosa* is on the increase.

1.2 BACTERIAL RESISTANCE TO ANTIMICROBIAL AGENTS.

Adaptive resistance derives from the bacterial chromosome and develops as a consequence of spontaneous mutations in a locus which controls susceptibility to an antimicrobial agent. The chromosomal mutants produced are often resistant by virtue of a change in a structural receptor for the drug. Acquired resistance is usually introduced by extra chromosomal genetic elements, commonly R plasmids, which often carry several genes confering resistance to one or several antimicrobial agents and heavy metals. This means that, in clinically important bacteria, plasmids tend to be the main reservoir of resistance to most antimicrobial agents (Jacoby 1986).

Many plasmid resistance genes have obvious chromosomal origins, either from genetically-distant species, as in aminoglycoside resistance (reviewed by Foster 1983); or from genetically-related species, as in trimethoprim resistance in *Staphylococcus aureus* (Iordanescu *et al* 1978) or the production of the β-lactamase BIL-1 (reviewed by Amyes *et al* 1992). A mechanism must exist, therefore, which enables the mobilsation of resistance genes from their original location to the plasmid. The discovery of transposons (Hedges and Jacob 1974) provided an explanation by which resistance genes could be moved independently to new sites within a genome, or be 'picked up' by a plasmid. Transposons are able to transpose genetic material from a donor DNA molecule to a recipient, but are unable to replicate themselves independently. Transposons, with a few exceptions, possess one, or several, transposase genes whose products act on inverted repeat sequences found at the ends of the transposable element (Berg 1989).

The modes of transfer of chromosomal and plasmid mediated resistance are fundamentally different; resistance determinants carried on the chromosome are normally restricted to vertical transmission, that is from mother to daughter cells during cell division and replication. Plasmids, however, may be transferred both vertically and horizontally, between bacteria of the same species or genera. Plasmid-mediated resistance thus tends to spread at a significantly faster rate than chromosomally-determined resistance.

Resistance to all antimicrobial agents can arise via four mechanisms, reviewed by Lewis (1989), which are:-

1) Alteration of the target site or hyperproduction of the target, thus reducing or preventing binding of the antimicrobial agent to the target.

2) Blockage of transport of the agent into the cell, usually by permeability changes.

3) Destruction or inactivation of the antibiotic.

4) Metabolic bypass - the cell is provided with a replacement for the metabolic step inhibited by the antimicrobial agent .

1.2.1 Alteration of the Target Site.

This type of resistance occurs as a result of the selection of rare, pre-existent mutants in a sensitive bacterial population, in the presence of an antimicrobial agent. Streptomycin resistance is brought about by a single amino acid mutation in the structural protein of the 30S ribosomal subunit of bacteria (Davies 1964). This mutation subsequently prevents drug binding and is responsible for high levels of drug resistance. Alterations in the 50S bacterial subunit may also bring about erythromycin resistance (Teroaka and Tanaka 1974); but generally erythromycin resistance in *staphylococci* and *streptococci* occurs because of the presence of an inducible plasmid mediated enzyme, which causes the methylation of the 23S ribosomal ribonucleic acid (rRNA) (Lai and Weisblum 1971). Resistance to a few penicillins and cephalosporins may be caused by mutations which encode the loss or alteration of penicillin binding proteins (PBPs) (Spratt 1983). Alterations in PBPs are very important resistance mechanisms in *Staphylococcus aureus*, *streptococci* and *enterococci,* as they prevent the inhibition of peptidoglycan synthesis, and the mutant bacteria are also resistant to imipenem.

1.2.2 Interference With Drug Transportation.

This resistance usually results from permeability changes encoded by mutations in the bacterial chromosome, which alter the size and/or the distribution of the membrane channels, thus preventing drugs from entering the cells. This affects a wide range of antimicrobial agents and generally results in cross resistance, where an organism becomes resistant to several unrelated antimicrobial agents at one time (Cohen *et al* 1988). Permeability mutations are particularly characteristic of Gram-negative bacteria such as *P.aeruginosa* (Hirai *et al* 1987) and *Escherichia coli* (Harder *et al* 1981).

Antibiotics affected by this type of resistance include aminoglycosides and tetracycline. Tetracycline resistance occurs in both Gram-positive and Gram-negative bacteria and is usually plasmid-encoded, although chromosomally-encoded tetracycline resistance has been reported (Foster 1983). Tetracycline resistance is associated with decreased accumulation of the drug, although the precise mechanism by which this occurs is unknown (McMurry *et al* 1980). Tetracycline resistance is generally inducible, and a new inner membrane protein is synthesised (Levy *et al* 1977) which is linked to the development of an active efflux system (McMurry *et al* 1980). This was demonstrated by the use of *E.coli* everted membrane vesicles which concentrated tetracycline by an active influx mechanism. Tetracycline-resistant cells exhibited no alteration in tetracycline accumulation compared to sensitive strains, demonstrating that the efflux of tetracycline is a major resistance mechanism, whereby drug entering the cell is simultaneously removed and never reaches an inhibitory level there (McMurry *et al* 1980).

1.2.3 Destruction or Inactivation of the Antibiotic.

The major cause of resistance to penicillins and cephalosporins is the production of β-lactamases, which catalyse the hydrolysis of the β-lactam ring to form an inactive product. In Gram-negative bacteria β-lactamase production may be either chromosomally- or plasmid-mediated. The β-lactamases that are chromosomally-mediated are normally closely bound to the cell membrane and are generally species-specific (Matthew *et al* 1975). Chromosomal β-lactamases also tend to be inducible, that is various different β-lactam antibiotics are able to stimulate the production of β-lactamases (Minami *et al* 1980). Chromosomally-mediated β-lactamases are found in most species of clinical bacteria.

In the clinical environment, resistance to penicillins and cephalosporins is caused by plasmid-mediated enzymes, of which more than 60 have been identified in Gram-negative bacteria (Wiedemann 1989). The most prevalent of these enzymes is designated TEM-1, and may be specified on a number of different plasmids (Roy *et al* 1983; Simpson *et al* 1986; Reid *et al* 1988), as the genetic information encoding it resides on a transposon. Transposons allow the translocation of genes between plasmids and the host chromosome, and probably explain the wide distribution of TEM-1 in the bacterial population, including many *Enterobacteriaceae, P.aeruginosa, Haemophilus influenzae* and *Neisseria gonorrhoeae.*

Plasmid-mediated resistance to aminoglycosides in Gram-negative bacteria is also brought about by drug inactivation. The plasmids encode modifying enzymes which either catalyse adenylylation, phosphorylation or acetylation reactions (Reynolds and Smith 1979; Shannon and Phillips, 1982). The modifying enzymes are present in the periplasmic space between the inner and outer cell membranes, and are closely associated with the inner cytoplasmic membrane where they are accessible to acetyl coenzyme A and adenosine triphosphate (ATP). The presence of a few molecules of the modified drug appear to interfere with active transport mechanisms and block further drug transport into the cell (Dickie *et al* 1978).

Drug inactivation is also responsible for bacterial resistance to chloramphenicol in both Gram-positive and Gram-negative bacteria (Shaw 1983). Resistance is plasmid encoded and is the result of acetylation of chloramphenicol by chloramphenicol acetyltransferases. The acetylated chloramphenicol can no longer bind to the bacterial ribosome and therefore has no effect on protein synthesis (Shaw 1967; 1971).

1.2.4 Metabolic Bypass.

This mechanism accounts for bacterial resistance to sulphonamides and trimethoprim, and occurs when the cell acquires a replacement for the metabolic step inhibited by the drug. In the case of the sulphonamides and trimethoprim, resistance is brought about by the production of target enzymes resistant to the antimicrobial agents in question.

Sulphonamides inhibit the enzyme dihydropteroate synthetase, which links para-aminobenzoate and pteridine to form dihydropteroate, a step in the synthesis of tetrahydrofolic acid, which is a co-factor essential in many of the metabolic processes of the living cell (reviewed by Foster 1983). In Gram-negative bacteria plasmids encode a sulphonamide-resistant dihydropteroate synthetase which co-exists with the chromosomal sulphonamide-sensitive enzyme in the cell, and thus permits the continued functioning of the metabolic pathway, even in the presence of the drug (Sköld 1976; Swedberg and Sköld 1980).

Trimethoprim acts at a later stage in the same metabolic pathway by blocking the action of the enzyme dihydrofolate reductase, which converts dihydrofolate into an active and reduced form, tetrahydrofolate. Trimethoprim-resistant bacteria produce a plasmid-encoded trimethoprim-resistant dihydrofolate reductase, which also co-exists in the cell with its chromosomally-encoded drug-sensitive counterpart (Amyes and Smith 1974; 1976). There are now over sixteen different plasmid-borne trimethoprim-resistant dihydrofolate reductases which have been differentiated by DNA:DNA hybridisation and biochemical characteristics (Amyes and Towner 1990).

1.3 THE QUINOLONE ANTIMICROBIAL AGENTS.

1.3.1 The History and Development of the Quinolones.

Unlike most of the antimicrobial agents in use today, the quinolones were not isolated from moulds and fungi; instead they are synthetically manufactured antimicrobials. The first quinolone, nalidixic acid (1-ethyl-1, 4-dihydro-7-methyl-4-oxo-1, 8-naphthyridine-3-carboxylic acid) was discovered as a by-product of antimalarial research in 1962, during the purification of chloroquine by Lesher and colleagues. Nalidixic acid was found to possess a narrow spectrum of activity against aerobic Gram-negative bacteria and was used primarily in the treatment of urinary tract and enteric infections, particularly against dysentery caused by *Shigella sonnei* (Moorhead and Parry 1965). It was subsequently discovered, however, that low level nalidixic acid-resistant organisms could be selected for easily *in vitro* and *in vivo* (Crook *et al* 1985), and it was assumed that nalidixic acid would run out of clinical usefulness.

During the 1970's two other quinolone compounds, oxolinic acid and cinoxacin, were developed, based upon the same general structure (Fig1.1). These too had a limited spectrum of activity against the *Enterobacteriaceae*.

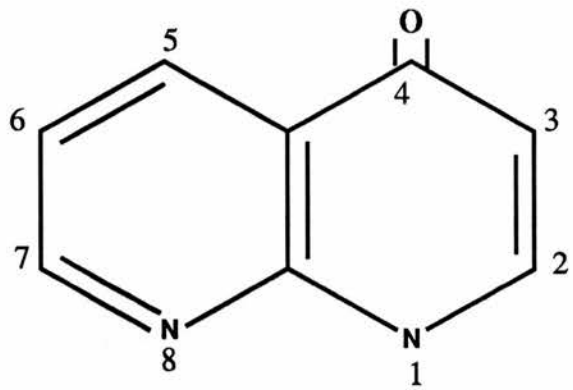


Fig 1.1. The Basic Quinolone Skeleton.

A breakthrough in quinolone research came with the introduction of a piperazine ring at position 7 and the addition of a fluorine to position 6 (Koga *et al* 1980; Rustige *et al* 1990). These substituents caused a thousand-fold improvement in the activity of the quinolones: the piperazine ring increasing the spectrum of activity of the drugs against P.*aeruginosa* and the nalidixic acid-resistant mutants of *E.coli* (Koga *et al* 1980). The first of the new fluorinated quinolones to be used clinically in Germany was norfloxacin, which had a broad spectrum of activity against Gram-negative and Gram-positive bacteria (Wolfson and Hooper 1989). Several other quinolone antimicrobials followed the development of norfloxacin, and these included ciprofloxacin, ofloxacin, pefloxacin and enoxacin. Most of the new quinolones are quinoline derivatives, whereas nalidixic acid and enoxacin with aza (nitro) substituents at position 8 of the basic quinolone structure are strictly naphthryidines. In order to avoid confusion the agents containing the basic quinolone skeleton were termed the 4-quinolones (Smith 1984a).

In all of the newer compounds the basic 4-quinolone nucleus with a carboxylate substituent at position 3 has been retained, with only side group substituents being changed (Fig 1.2). The similarity in the structures of the new 4-quinolone compounds occur as the 4-quinolone structure has been shown to be relatively fixed. Schentag and Domagala (1985) demonstrated that in order for quinolones to interact with their target, bacterial DNA gyrase, the possession of a carboxylic acid at position 3 and a β-ketone at position 4 were essential. Also, the possession of a fluorine molecule at position 6 is necessary for a broad antimicrobial spectrum and high activity (Koga *et al* 1980). Antipseudomonal activity is further enhanced by having a nitrogen grouping at position 7 (Rustige *et al* 1990). Modification of position 2 by the addition of any group was shown to lead to complete loss of antimicrobial activity.

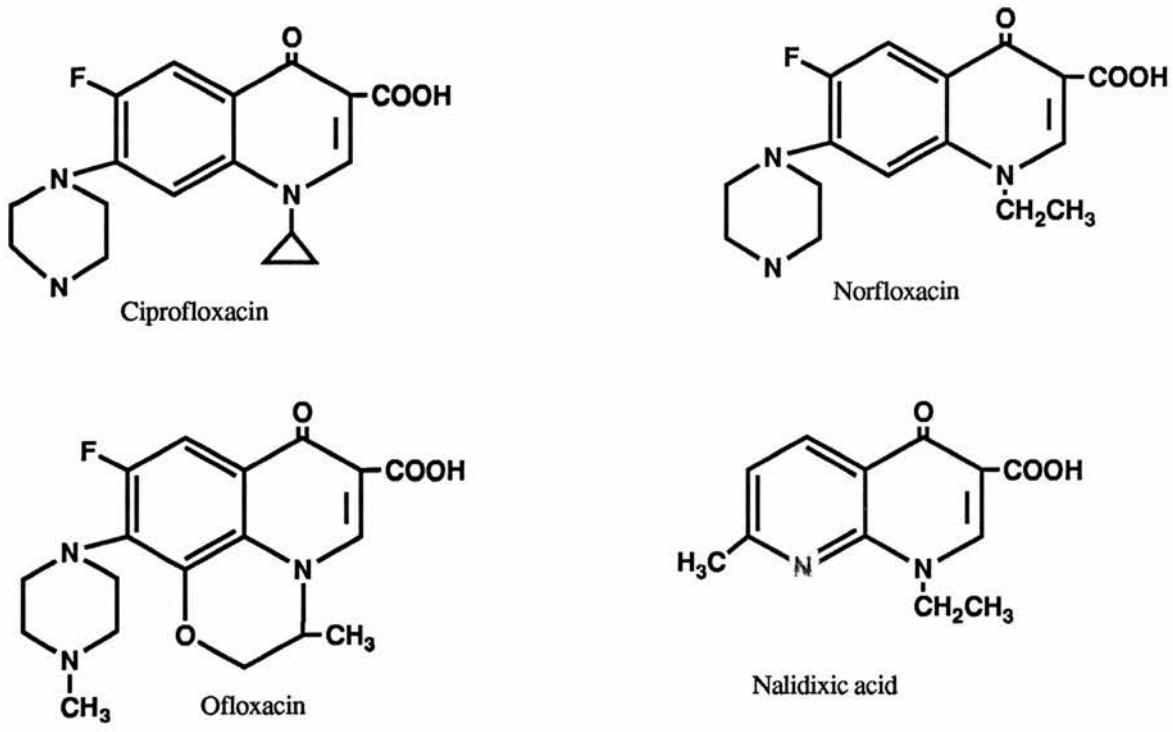


Fig 1.2. Structures of the New 4-quinolones Compared to Nalidixic Acid.

The more recent 4-quinolones, such as temafloxacin, have a modified position 1 which includes a phenyl group that has increased the solubility of the compound. Modification of the piperazine ring has also improved the lipid solubility of temafloxacin compared with ciprofloxacin and ofloxacin (Percival 1991).

In comparison with nalidixic acid the fluorinated quinolones have an extended spectrum of microbiological activity. These quinolones are most active against *E.coli, Shigella, Salmonella, Enterobacter* and *Neisseria* species. They are moderately active against *P.aeruginosa, H.influenzae* and *Legionella pneumophila* and have low activities against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) (Wolfson and Hooper 1985).

The recently developed 4-quinolones, temafloxacin and WIN 57273, show a greater antimicrobial spectrum for Gram-positive organisms such as *S.aureus, Streptococcus* and *Enterococcus* species. This increase in apparent potency against Gram-positive bacteria and obligate anaerobes, which were previously intrinsically resistant to the older 4-quinolones, was brought about by the addition of a second fluorine molecule to the basic structure.

In clinical use the fluorinated quinolones are characterised by improved absorption, wide distribution into body tissues, and lower half lives in the body than nalidixic acid. They are used mainly in the treatment of urinary tract infections and also as a second line treatment for lower respiratory tract, bone and joint infections. Quinolones are also used as a second line treatment where bacterial resistance to primary drug treatments has developed; and have been seen to be active *in vitro* against multiply antibiotic resistant Gram-negative bacilli and β-lactamase producing *N.gonorrhoeae* (Wolfson and Hooper 1985).

1.3.2 Quinolone Uptake Into Bacterial Cells.

Uptake into bacterial cells is thought to be important in determining the activity and antibacterial spectrum of the quinolones, and decreased drug uptake is thought to play a role in quinolone resistance. The outer membrane is an effective barrier to drug permeation in Gram-negative bacilli, and generally there are three routes by which antimicrobial agents may enter a cell; the porin pathway, the hydrophobic pathway and the self promoted route utilised by cationic compounds (Bryan and Bedard 1991).

At first quinolones are rapidly taken up by bacterial cells and then uptake reaches a steady state; in the *Enterobacteriaceae* and *S.aureus* this state is reached after 60-80 seconds, whereas *P.aeruginosa* and *Klebsiella pneumoniae* achieve a steady state plateau at six minutes (Piddock 1991). Uptake in *P.aeruginosa* and *E.coli* has been shown to be non-saturable (Bedard *et al* 1987; Bedard *et al* 1989; Celesk and Robillard 1989; Hooper *et al* 1989), which indicates that uptake probably occurs by simple diffusion through non-specific channels and not by saturable carrier proteins. Experiments by Hirai and colleagues (1986b) provided strong evidence that in *E.coli* all quinolones enter the cell via the porin pathway.

Other factors implicating passive diffusion through water filled porin channels include experiments by Bedard and co-workers (1987), which showed no competition between uptake of ciprofloxacin or enoxacin in *E.coli* and *Bacillus subtilis*. Also, mutants of *E.coli* and *Salmonella typhimurium* exhibiting decreased expression of a membrane porin, outer membrane protein F (OMPF), show resistance to and a decreased uptake of quinolones. (Hirai *et al* 1986a; Bedard *et al* 1987; Cohen *et al* 1989).

Quinolones may also penetrate the outer membrane via the phospholipid bilayer. Hirai and colleagues (1986a) demonstrated that quinolones with low relative hydrophobicity, like ciprofloxacin, penetrate through OMPF in *E.coli* and *S.typhimurium*, but those with high relative hydrophobicity, such as nalidixic acid and oxolinic acid, permeate through OMPF and the phospholipid bilayer. Nalidixic acid and oxolinic acid also exhibited decreased uptake in *S.typhimurium* strains with lipopolysaccharide (LPS) defects compared with the newer fluorinated quinolones.

Quinolones have been shown to exhibit self-promoted uptake by acting as chelating agents for divalent cations on the cell surface, and so facilitating the entry of other quinolone molecules (Chapman and Georgopapadakou 1988). Fleroxacin and the newer 4-quinolones are able to chelate magnesium ions on the cell surface LPS, causing it to be displaced and creating hydrophobic patches of exposed lipid domains on the outer membrane. This then enables other fleroxacin molecules to diffuse through these exposed domains.

Chapman and Georgopapadakou's (1988) observations were supported by the work of Smith and Lewin (1988), which showed that the presence of magnesium ions could increase bacterial minimum inhibitory concentrations (MICs) of quinolones. Chapman and Georgopapadakou (1988) also demonstrated that diffusion rates through the bacterial outer membrane were influenced by the relative hydrophobicity and ionic charge of the quinolones, and that the preferred route of penetration for enoxacin, ciprofloxacin and norfloxacin, was through the porin channels. The porin pathway may also be the preferred route for fleroxacin penetration (Hirai *et al* 1986a), but failure to detect fleroxacin-resistant mutants with only OMPF defects indicates that the non-porin pathway is also a major entry route for this agent (Chapman *et al* 1989).

Self-promoted uptake of quinolones has not been shown to occur in *P.aeruginosa* (Young and Hancock 1992). Strains of *P.aeruginosa* overexpressing a non-porin protein, *OprH*, were shown to exhibit increased quinolone uptake into the cell, in comparison with parent strains. Overexpression of *OprH* is thought to neutralise the overall negative charge on the cell surface of *P.aeruginosa*, as it is a basic protein, and allows quinolones better access to the sites involved in uptake via the non-porin pathway.

Studies of the passage of 4-quinolones through the inner membrane of Gram-negative bacteria have led to conflicting results. Chapman and Georgopapadakou (1988) advocate a passive diffusion mechanism, which is therefore energy independent, and this has been corroborated by Bedard and colleagues (1987) with their studies on enoxacin uptake in *E.coli* and *Bacillus subtilis.* An energy-dependent active transport mechanism, however, has also been implicated, coupled to the proton motive force, as the uptake of ciprofloxacin, pefloxacin and amifloxacin were inhibited by 2,4-dinitrophenol (DNP), an uncoupling agent (Diver *et al* 1990b).

Whatever the pathway of quinolone uptake into bacterial cells, whether by an active or totally passive means, it is clear that the cell wall of quinolone sensitive bacteria provides very little interference to drug accumulation in the cell. This is because 4-quinolone uptake, bactericidal activity, and inhibition of DNA synthesis are very rapid. It is also clear from studying resistant mutants that no single mechanism accounts for the total quinolone uptake into the bacterial cell, although the porin pathway seems to be the preferred route.

1.4 MODE OF ACTION OF QUINOLONES.

The quinolones have been shown to act upon a unique bacterial enzyme, DNA gyrase (bacterial topoisomerase II : EC number 5.999.1.3) (Gellert *et al* 1976). DNA gyrase has the unique ability to introduce negative supercoils into closed circular double stranded DNA with adenosine triphosphate (ATP) hydrolysis. DNA gyrase is essential for bacterial function, and in the absence of ATP is able to relax negative supercoiled DNA. DNA gyrase is also responsible for catenating and decatenating duplex DNA circles, and resolving knotted single DNA duplexes. In the presence of ATP, DNA gyrase relaxes positively supercoiled DNA in a reaction analogous to the introduction of negative supercoils.

Bacteria are faced with a major problem, as the DNA which encodes their characteristics is a large linear molecule. In *E.coli* the DNA is 1,100 μm in length (Cairns 1963) and must be accommodated in a cell of approximately 1 or 2 μm in length. This begged the question of how bacteria managed to accommodate the DNA in the cell. In 1974 Worcel found the answer when studying the *E.coli* chromosome. He discovered that the *E.coli* chromosome is divided into about 65 separate regions, or domains, of 20 μm in length, each domain being attached to a ribonucleic acid (RNA) core. The size of each domain was also reduced by supertwisting against the direction of the DNA helix, termed negative supercoiling (Wang 1974). The introduction of negative supercoils reduces the number of helical turns required in the DNA, thus compacting it, and also aids strand separation.

DNA also presents bacteria with another problem. In order to survive bacteria must replicate, which involves the duplication of DNA, its division into daughter chromosomes, the transcripton of genes to form new cell components, and the splitting into two daughter cells without lethally entangling the DNA present. The nature of the DNA helix is such that in *E.coli* the helix, comprised of $4x10^6$ base pairs, is intertwined 400,000 times, and this must unwind 400,000 times in order for semiconservative replication to occur. To bring about unwinding, the strands separate and a 'swivel' mechanism operates in front of the replication fork as it advances (Cairns 1963). The negative supercoiling of DNA also provides the bacteria with a unique problem, as energy is required to both supercoil and uncoil the DNA.

In prokaryotes the condensing and replicating of DNA is carried out by topoisomerases (Gellert *et al* 1976). Topoisomerases are present in every cell and are enzymes responsible for altering the linking number of DNA by controlling, via the breaking and rejoining of strands, the number of times the duplex winds around its complementary strand. Topoisomerases are divided into two main groups:-

1) Type I are responsible for relaxing negatively supercoiled DNA, conversion of two closed single strands of DNA into a double stranded form, knotting and nicking single stranded DNA and the interlocking of DNA duplexes (Rose 1988). They reduce the linking number by one.

2) Type II topoisomerases alter the linking number by two, and are able to unknot and catenate unnicked DNA, and may break both DNA strands. Some type II topoisomerases are able to introduce positive or negative twists into DNA, and most type II topoisomerases require ATP as a co-factor to catalyse reactions (Sutcliffe *et al* 1989).

1.5 THE STRUCTURE AND FUNCTION OF DNA GYRASE.

DNA gyrase is a type II topoisomerase and is unique to prokaryotes. It is the only enzyme known that is able to introduce negative supercoils into covalently closed circular DNA; a reaction which requires adenosine triphosphate (ATP). DNA gyrase has been isolated from many bacterial species including *E.coli* (Swanberg and Wang 1987), *Micrococcus luteus* (Klevan and Wang 1980), *P.aeruginosa* (Robillard and Scarpa 1988), *Citrobacter freundii* (Aoyama *et al* 1988a), *B.subtilis* (Lampe and Bott 1984) and *Streptomyces sphaeroides* (Thiara and Cundliffe 1988), and has been found to possess a highly conserved structure. This is highlighted further by the cloning and sequencing of the *gyrA* genes of *E.coli* (Swanberg and Wang 1987), *B.subtilis* (Lampe and Bott 1984), *S.aureus* (Hopewell *et al* 1990), *Klebsiella pneumoniae* (Dimri and Das 1990) and *C.freundii* (Aoyama *et al* 1988b), in which greater than 50% homology is seen. This high degree of homology is both structural and functional as the *gyrB* subunit of *B.subtilis* has been demonstrated to complement the *gyrA* of *E.coli in vitro* to form an active gyrase complex (Orr and Staudenbauer 1982).

DNA gyrase is a tetrametric molecule composed of two α and two β subunits. The gyrase complex was demonstrated to contain equivalent amounts of α and β proteins by mixing different ratios of the separate gyrase proteins with DNA, followed by the separation of the DNA-protein complex from the free protein by gel filtration (Sugino *et al* 1980). Sedimentation analysis of gyrase complexed with 140 base pair fragments of DNA suggested that the gyrase had an $\alpha_2\beta_2$ composition (Klevan and Wang 1980). This structure was further supported by small angle neutron scattering, suggesting a mass of 353 kilodaltons (kD) for the *E.coli* DNA gyrase which is compatible with an $\alpha_2\beta_2$ complex (Kreuger *et al* 1990). In *E.coli* the α subunits are 97kD proteins encoded by the *gyrA* gene, located at 48 minutes on the *E.coli* map, and the β subunits are 90kD in mass, encoded by the *gyrB* gene and located at 83 minutes on the *E.coli* map. In *B.subtilis* the *gyrA* and *gyrB* genes are contiguous and located in the region of the replication origin.

The action of DNA gyrase has been well studied (Horowitz and Wang 1987, Morrison and Cozzarelli 1981). The enzyme binds non-covalently to sequences of DNA; in *E.coli* this DNA binding site was shown to be at the amino acid tyrosine 122 on the α subunit (Horowitz and Wang 1987). The binding sites on the DNA, however, show no specific sequence, although there is a general consensus (Fisher *et al* 1981). DNA gyrase then requires co-factors of magnesium ions and ATP to introduce negative supercoils (Higgins and Cozzarelli 1982). The requirement for ATP is specific, as dATP is a poor substitute and other nucleotide triphosphates are completely inactive (Sutcliffe *et al* 1989).

In 1981 Morrison and Cozzarelli proposed a model for negative supercoiling by gyrase. Double stranded DNA is wrapped around the outside of the gyrase with a 50 base pair central region being protected by the gyrase. The α subunit is responsible for nicking each strand of the DNA helix at intervals of four base pairs apart, producing a staggered cut, and then holds the strands apart (Fig 1.3). The DNA strand is then passed through the nicks in a positive sense, introducing an extra twist, by the β subunit. The α subunits then reseal the nicked strands, restoring the DNA helix. The binding of ATP induces a conformational change in the gyrase-DNA complex, triggering strand passage through the nick in the helix, and is then hydrolysed to 'recock' the gyrase-DNA complex, and another cycle is initiated (Rau *et al* 1987).

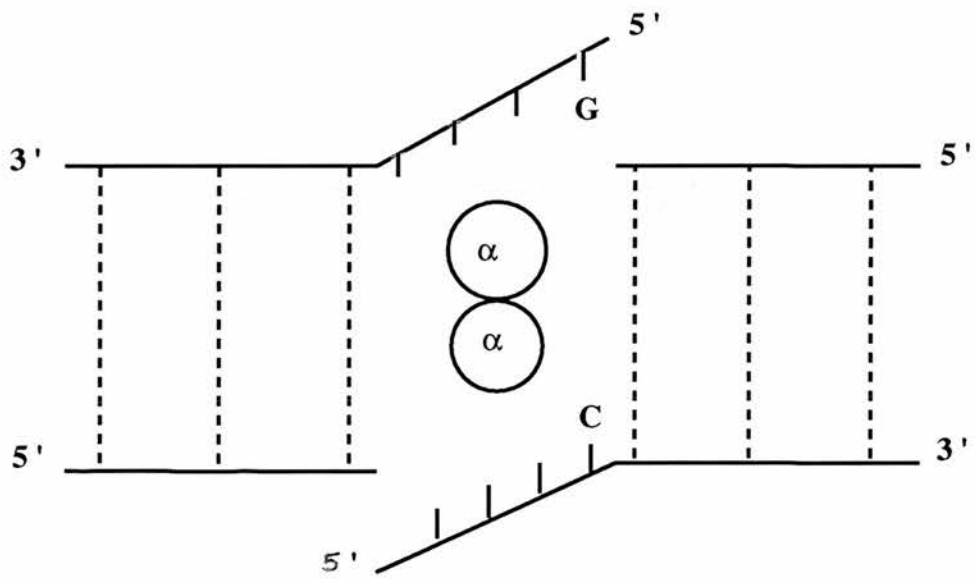


Fig 1.3. The α-Subunits of DNA Gyrase Introduce Nicks Into the DNA Strands at Specific Sites 4 Base Pairs Apart (Source: Bayer, UK).

In the bacterial cell DNA gyrase and topoisomerase I act in concert to regulate DNA transcription and replication. The amount of each enzyme is dictated by the superhelicity of the DNA molecule: decreasing the negatively supercoiled state of DNA stimulates the transcription of *gyrA* and *gyrB,* and suppresses the transcription of the *topA* gene. Increasing the superhelicity of DNA has the opposite effect, repressing *gyrA* and *gyrB* transcription whilst inducing transcription of *topA* (Menzel and Gellert 1983).

1.6 INHIBITION OF DNA GYRASE.

The action of DNA gyrase is inhibited by two major classes of antimicrobials, the courmarins which include novobiocin, and the quinolones.

1) **Courmarins** : These inhibit bacterial DNA, RNA and protein synthesis, with their primary effect exerted on DNA synthesis (Ryan 1976). Investigations by Gellert *et al* (1976) showed that novobiocin and coumermycin caused the relaxation of negative supercoils, and, by utilising drug-resistant bacteria, demonstrated that the target of the courmarins is the gyrase β subunit. The courmarins act by binding directly to the ATP binding site on the β subunit and prevent ATP hydrolysis.

2) **Quinolones** : the target for the quinolones in the bacterial cell was determined to be the α subunit of DNA gyrase, when Sugino and colleagues (1977) purified the *nalA* gene and showed it to be the target for nalidixic acid. In the same year the α subunit of DNA gyrase was proved to be the primary target of oxolinic acid and nalidixic acid (Gellert *et al* 1977). Earlier investigations had indicated this when Goss (1964;1965) and his co-workers (Cook *et al* 1966; Dietz *et al* 1966) reported that nalidixic acid selectively antagonised DNA synthesis, caused DNA degradation, and induced bacterial filamentation.

Other evidence that the α subunit was the target for 4-quinolones was that :-

a) Quinolones are highly specific inhibitors of DNA gyrase.

b) Mutations leading to high level drug resistance are found predominantly in *gyrA*, the structural gene for the α subunit.

c) Quinolones inhibit the supercoiling activities of DNA gyrase, the active site of which is in the α subunit.

Despite many investigations, it has been impossible to elucidate the mechanism by which quinolones exert their effects. Initial reports suggested that quinolones bound directly to DNA (Shen and Pernet 1985), as tritiated norfloxacin was demonstrated to bind preferentially to single stranded DNA and not to DNA gyrase, and on binding, norfloxacin blocked the functions of DNA gyrase. Other reports directly opposed this view, suggesting that DNA gyrase was the target for the quinolones, as quinolones are able to inhibit the activity of purified DNA gyrase (Barret *et al* 1990). Also, ^{19}F nuclear resonance experiments by Le Goffic (1985) demonstrated the affinity of pefloxacin for DNA gyrase. Further evidence for quinolone binding to DNA gyrase comes from bacterial resistance associated with DNA gyrase mutations (Gellert *et al* 1977; Sato *et al* 1986).

Another model for the molecular basis of quinolone action was proposed by Shen *et al* (1989). They suggested that a co-operative drug-DNA binding model occurred, which inhibited gyrase. In this model DNA gyrase binds to double stranded DNA, cleaves both DNA strands and induces a binding site for the quinolones on the relaxed DNA. Drug binding occurs on the exposed single strand region at the enzyme's active site, prior to the DNA strand being twisted through the DNA 'gate', and binding locks the DNA strands in place, preventing the turnover of the gyrase molecule.

Quinolone binding to DNA is proposed to occur by hydrogen bonds formed between the DNA and the carbonyl and carboxyl side chains common to all quinolones. Drug binding is thought to be co-operative with the binding of one quinolone molecule facilitating the binding of a second, with four or more molecules binding in the DNA 'gate' and interacting with each other by ring stacking.

This proposed model is further supported by experiments performed by Willmott and Maxwell (1993), who found by rapid gel filtration techniques that norfloxacin bound to a gyrase-DNA complex, and not to the gyrase or DNA alone; and that the efficiency of quinolone binding was determined primarily by the *gyrA* subunits. Yoshida and co-workers (1993) dispute this model, contending that a quinolone pocket model is favoured, as the quinolone stacking model takes no account of mutations in *gyrB* causing high level resistance, or the fact that quinolones have a wide variety of side chains and structures that DNA is meant to bind to. The pocket model proposes that quinolones exert their action by binding to gyrase-DNA complexes, and that the binding affinities of the quinolones for the complex is determined by both the *gyrA* and *gyrB* subunits. Yoshida and colleagues (1993) also demonstrated that enoxacin exhibits two different binding affinities for the gyrase-DNA complex, depending upon the mutant strain used, which also supports the pocket model theory. It is still unclear, however, whether quinolones make contact with the α subunit alone, or with the β protein as well, as mutations in both the *gyrA* and *gyrB* genes of DNA gyrase have been demonstrated to reduce quinolone binding.

1.7 ACTIONS OF QUINOLONES ON INTACT BACTERIA.

As DNA gyrase inhibition prevents bacterial replication, the effect of the quinolone antimicrobials might be assumed to be bacteriostatic. This, however, is not the case, as exposure of susceptible bacterial cells to all quinolones results in rapid cell death (Smith 1984a; Lewin and Hastings 1990). Subjecting susceptible bacteria to quinolone concentrations above the MIC leads to a biphasic response. In the first instance, the lethality of the drugs increases until an optimum bacterial concentration (OBC) is reached, and thereafter bactericidal activity declines.

One reason suggested for this biphasic response is that the 4-quinolones inhibit bacterial RNA synthesis at concentrations above the OBC (Smith 1984a; Smith and Lewin 1988). The inhibition of DNA gyrase may cause the relaxation of chromosomal DNA to such an extent that it can no longer be efficiently transcribed into RNA. Smith and Lewin (1988) established that bacterial protein and RNA synthesis are prerequisites for the bactericidal activity of nalidixic acid in *E.coli* by abolishing nalidixic acid's bactericidal activity with the addition of rifampicin, an inhibitor of bacterial RNA synthesis. Thus, the inhibition of RNA synthesis above the OBC would be expected to abolish the bactericidal activity of the quinolones.

The bactericidal activity of nalidixic acid, however, does not explain the killing mechanisms of all the 4-quinolone antimicrobials. Whilst the addition of a protein synthesis inhibitor, chloramphenicol, or rifampicin, an inhibitor of RNA synthesis, completely inhibits the bactericidal activity of nalidixic acid against *E.coli* in nutrient broth, they only reduce the bactericidal activity of ciprofloxacin and norfloxacin under the same conditions (Smith 1984a; Smith and Lewin 1988). A second observation was that nalidixic acid does not exhibit a lethal effect in phosphate buffered saline, where bacteria are unable to divide, whereas both ciprofloxacin and ofloxacin are active in this medium (Smith 1984b).

These results led to the proposition that the principal mechanism of action of all the 4-quinolones was a mechanism which required protein and RNA synthesis, and cell division for lethality, and this mechanism, mechanism A, was the only bactericidal mechanism exhibited by nalidixic acid. Smith (1984a) also suggested that ciprofloxacin and ofloxacin possessed a second bactericidal mechanism, termed mechanism B, which was independent of protein and RNA synthesis and active against non-dividing bacteria. Mechanism B occurs in the newer flouroquinolones like lomefloxacin (Lewin *et al* 1989a).

Not all quinolones exhibit mechanism B. Investigations into the action of norfloxacin demonstrated that the bactericidal activity of the drug was abolished in the absence of protein or RNA synthesis, depending upon whether chloramphenicol or rifampicin was added to the medium. Norfloxacin, unlike nalidixic acid, is active against non-dividing bacterial cells, and therefore displays a third mechanism of action, termed C, in addition to mechanism A (Ratcliffe and Smith 1985). Enoxacin also exhibits mechanism C (Lewin *et al* 1989a). The possession of an extra bactericidal mechanism on top of mechanism A may explain why the fluoroquinolones are more bactericidal at their respective OBC's than nalidixic acid.

Inhibition of protein and RNA synthesis may reduce or abolish the ability of the quinolones to kill bacteria, but it has no effect on their ability to prevent DNA synthesis (Dietz *et al* 1966). Other conditions may also affect the bactericidal activity of quinolones : strict anaerobiosis (Morrissey *et al* 1990), treatment with dinitrophenol (DNP) (Dietz *et al* 1966), and starvation in buffered saline (Lewin *et al* 1989b) all reduced the bactericidal activity of the quinolones studied. The quinolones may also exhibit different bactericidal mechanisms against different species of bacteria. Ciprofloxacin has been shown to kill *E.coli* by mechanism B, but during preliminary

investigations seems to lack mechanism B against *Staphylococcus albus* or *S.aureus* (Lewin and Smith 1987).

Against *E.coli* and *S.aureus,* ciprofloxacin and ofloxacin have been found to be bacteriostatic under anaerobic conditions (Smith and Lewin 1988; Lewin *et al* 1989b, Morrissey *et al* 1990). Aeration of cultures was shown to restore some bactericidal activity and it was postulated that DNA supercoiling may account for the lack of effect. DNA superhelicity has been implicated as a control mechanism in the expression of several genes involved in the anaerobicity of both *E.coli* and *Salmonella typhimurium* (Yamamoto and Droffner 1985; Dorman *et al* 1988). Mutants of *S.typhimurium* which were obligate aerobes were found to be gyrase-deficient, implying that gyrase activity is essential for growth under anaerobic conditions. Anaerobicity is also responsible for influencing DNA supercoiling, and through this regulates the production of the OMPC porin gene (Dorman *et al* 1988). Lewin and colleagues (1989b) postulated that altered porin configuration might lead to a decrease in quinolone uptake, and so decrease their bactericidal activity under anaerobic conditions.

Although the exact method of cell death caused by quinolones is still unclear, several proposals have been put forward. Smith (1984a) proposed that quinolones cause DNA gyrase to damage DNA by nicking, thus inducing the SOS DNA repair system. The SOS genes code for a cascade of proteins which repair DNA, and may be induced by the 4-quinolones (Piddock and Wise 1987). The quinolones also triggered the SOS response with maximum intensity at a drug concentration which produced the greatest bactericidal effect, and it was proposed that one of the SOS genes might code for a lethal protein (Phillips *et al* 1987). Later work has implicated the SOS gene *sfiA* in the lethality of the 4-quinolones (Walters *et al* 1989).

Other investigations have indicated that mutations which lead to defective SOS responses have an effect on, or increase, the susceptibility of bacteria to nalidixic acid and ciprofloxacin, and that the induction of the SOS response is purely consequential (Lewin *et al* 1989c; Piddock *et al* 1990). These results contradict the proposal that an SOS gene encodes a lethal protein, and it was suggested that the hypersusceptibility of some SOS deficient mutants is a result of deficient recombination repair and not SOS repair (Lewin *et al* 1989c). The induction of the SOS response and its subsequent effects fits the criteria of killing by mechanism A proposed by Smith (1984a). Quinolones induce the production of *recA* , the primary protein in the SOS response, and this induction requires active protein synthesis, as does mechanism A. The inhibition of cell filamentation at concentrations above the OBC, and the subsequent inhibition of cell division, also fit the criteria for mechanism A (Piddock *et al* 1990). The bactericidal activity of quinolones against mutants with defective SOS responses would then presumably be caused by mechanism B.

The use of electron microscopy techniques has demonstrated that quinolones alter the membrane integrity of *E.coli*, resulting in cytoplasmic leakage and ultimately cell death (Dougherty and Saukkonen 1985). Chapman and Georgopapadakou (1988) reported that quinolones induced LPS release from cell surfaces and acted as chelating agents for membrane bound divalent cations. The chelating effect lead to membrane disorganisation, which then facilitated the entry of further quinolone molecules through the outer membrane.

Other effects mediated by quinolones are cell filamentation and plasmid elimination. Filamentation has been reported in both *P.aeruginosa* (Benbrook and Miller 1986) and *E.coli* (Crumplin *et al* 1984) when cells were treated with norfloxacin, and is indicative of an imbalance in normal cell metabolism. The inhibition of DNA gyrase by norfloxacin may result in several effects : premature cell division, delayed cell division

events, and total failure of cell division culminating in lysis of the cell (Crumplin *et al* 1984).

Investigations on *E.coli* filamentation, utilising ciprofloxacin, determined that gross filamentation occurred at concentrations close to the MIC, but that filamentation was not as extensive at the most bactericidal concentration (Diver and Wise 1986). This was a result of inhibition of RNA and protein synthesis; and cell death caused by filamentation was found to be a secondary effect as 90% of the cells were non-viable. The secondary effect of filamentation did, however, eliminate around 10% of the cells, which concurs with Smith's postulate (1984a) that ciprofloxacin exhibits two mechanisms of killing in *E.coli*. In all cases, filamentation occurs before cell death and may be a result of the antagonism of DNA synthesis and the induction of the SOS response (Phillips *et al* 1987).

Plasmid elimination was reported in *E.coli* on treatment of bacterial cells with subinhibitory concentrations of nalidixic acid and fluoroquinolones (Weisser and Wiedemann 1985; 1986). Platt and Black (1987) also established this phenomenon when treating *E.coli* with ciprofloxacin, but found that only R plasmids were eliminated and not small high copy number plasmids. Further studies of an *E.coli* mating system suggested that some quinolones could reduce the efficiency of plasmid conjugation at quinolone concentrations at or above the MIC (Weisser and Wiedemann 1987). Furthermore, treatment with quinolones, particularly enoxacin, has been found to affect plasmid function, resulting in a decrease in antibiotic resistance gene expression and in replication related transcription (Courtright *et al* 1988). Whilst these observations offer no insight into the bactericidal mechanisms of the quinolones they might help to explain the lack of plasmid mediated resistance to these antimicrobial agents.

1.8 BACTERIAL RESISTANCE TO THE FOUR-QUINOLONES.

As described previously there are several mechanisms of bacterial resistance to antimicrobial agents, and these fall into two major categories, plasmid and chromosomally mediated resistance. Quinolones, unlike most other antimicrobial agents, have no substantiated reports of plasmid mediated resistance. Two reports of plasmid mediated resistance to quinolones have occurred, however, the first by Munshi and colleagues (1987) in *Shigella dysenteriae* and the second by Tanaka *et al* (1991) in *S.aureus.*

Munshi and colleagues (1987) claimed that *S.dysenteriae* carried a 20 megadalton plasmid encoding nalidixic acid resistance which could be transferred by conjugation. Their claim was refuted after careful inspection of the data, as it was established that nalidixic acid resistant mutants of the recipient strain had been selected for and not studied, and not the authentic transconjugants (Courvalin 1990). Tanaka and colleagues' report (1991) is slightly more conclusive, but the strains carrying the alleged plasmid borne resistance also possessed *gyrA* mutations, and it is likely that the methods of plasmid extraction from these strains could have caused the changes in MIC observed and not plasmid borne resistance (Tanaka personal communication). The absence of plasmid-mediated resistance to quinolones was confirmed by extensive experiments conducted by Burman (1977) who attempted, unsuccessfully, to transfer nalidixic acid resistance into *Proteus mirabilis, Klebsiella, Enterobacter* and *Pseudomonas* species.

Other evidence which suggests that plasmid-mediated quinolone resistance is unlikely was submitted by Weisser and Wiedemann (1987) who established that quinolones have a plasmid curing effect in *E.coli*. Plasmid conjugation is also inhibited by quinolones (Nakamura *et al* 1976; Burman 1977) and expression of mutants causing decreased outer membrane permeability is delayed when introduced into wild-type cells (Foulds 1976). This might indicate that quinolone resistance determined by outer membrane protein alterations may also be delayed.

A plasmid-mediated drug destruction mechanism is unlikely to occur, as quinolones are synthetic drugs. However, bacteria have developed an efflux system for 4-quinolones which probably evolved from an existing efflux mechanism. Therefore, a remote possibility exists that bacteria could adapt a plasmid-mediated drug modification mechanism to suit 4-quinolones.

A plasmid-borne, resistant DNA gyrase is also unlikely as resistant gyrases have been shown to be recessive to the sensitive wild-type gyrase gene in genetic studies (Hane and Wood 1969; Yoshida *et al* 1988; Robillard 1990). This trait was employed to identify alterations in DNA gyrases of resistant Gram-negative bacteria by conferring quinolone sensitivity to those strains by transforming them with plasmids carrying quinolone-susceptible genes (Lewin *et al* 1990).

1.8.1 Chromosomally Mediated Resistance to the Four-Quinolones.

Chromosomally-mediated resistance to the 4-quinolones is the only confirmed resistance mechanism reported, and may occur in several ways: mutations may occur that affect the permeability of the drug into the bacterial cell, whether by LPS or porin alterations. Also, there may be alteration in the target molecule, DNA gyrase. A third mechanism is the active efflux of the drug from the cell. In the case of the quinolones all three mechanisms of resistance have been reported, and will be dealt with separately.

1.8.1.1 ALTERED PERMEABILITY.

Resistance to quinolones via altered drug permeability has been documented for *E.coli* (Hirai *et al* 1986a; 1986b) *K.pnemoniae* (Sanders *et al* 1984), *Enterobacteriacae* and *Serratia* species (Gutmann *et al* 1985), *P.aeruginosa* (Hirai *et al* 1987; Inoue et al 1987), *Proteus vulgaris* (Ishii *et al* 1991) and *Citrobacter* species (Aoyama *et al* 1988a). Most reports conclude that this resistance is brought about by changes in outer membrane proteins, or porins, although some reports have also implicated LPS changes in reduced uptake (Hirai *et al* 1986b; Moniot-Ville *et al* 1991). The resistance to quinolones via altered permeability mechanisms has been well documented in *E.coli,* and for this reason *E.coli* will be concentrated upon here.

Quinolone resistance brought about by altered permeability is generally characterised by low level resistance to the quinolones and structurally unrelated drugs. High level resistance linked to permeability changes, however, have been documented in *Citrobacter freundii* and *Bacillus fragilis* (Kato *et al* 1988). In *E.coli* several mutations have been established (Table 1.1). The first report of low-level nalidixic acid resistance was by Hane and Wood (1969) when investigating *E.coli* K-12, and was designated *nalB*. Later in 1973, Bourguignon and colleagues identified *nalB* as a permeability mutation, as EDTA abolished the resistance to nalidixic acid. Other mutations involving low level nalidixic acid are *icd, purB* and *ctr* whose mechanisms of action are

unknown. The mutations *crp* and *cya* occur in the cyclic adenosine monophosphate (cAMP) genes and are responsible for pleiotropic drug resistance to quinolones and aminoglycosides, brought about by a decreased cAMP-dependent OMPF expression.

Cohen and co-workers (1989) found that quinolone resistance could be selected with tetracycline or chloramphenicol. This cross resistance was a result of changes in two outer membrane proteins, OMPC and OMPF. Cohen and colleagues (1989) speculated that drug permeability was decreased as a result of the increased expression of a narrow channelled porin, OMPC, coupled with decreased expression of the wider channelled porin, OMPF.

GENOTYPE	MECHANISM AND LINK	LOCATION (mins)
cfxB	marA energy dependent decrease in drug accumulation	32
nfxB	marA energy dependent decrease in drug accumulation	19
nalB	low level resistance	58
norB	decreased expression of OMPF porin	34
norC	decreased expression of OMPF porin, defective LPS core, hypersusceptibility to detergent	8
cya	decrease in cAMP dependent OMPF expression	85
crp	decrease in cAMP dependent OMPF expression	74
ctr	-	52
icd, purB	-	25

Table 1.1. Permeability Mutations in *Escherichia coli.*

The importance of OMPF expression in quinolone resistance was established by Hirai and colleagues (1986b), who documented that spontaneous single-step *E.coli* mutants deficient in OMPF were four-fold less susceptible to quinolones than the parent strain, and also accumulated norfloxacin at a slower rate. OMPC mutants were shown to accumulate norfloxacin at the same rate as the parent strain, suggesting that OMPF is the major route for influx of quinolones into *E.coli* (Hirai *et al* 1986b). These results have been confirmed by other authors. Hooper *et al* (1989) isolated two mutations *nfxB* and *cfxB*, selected with norfloxacin and ciprofloxacin, respectively. Both mutations mapped at sites distinct to the OMPF protein, which maps at 21 minutes. Both *nfxB*, mapping at 19 minutes, and *cfxB*, mapping at 34 minutes, are mutations in the regulatory genes which control OMPF expression at the post-transcriptional level, and cause a major decrease in OMPF expression (Lewin *et al* 1990).

CfxB was found to be closely linked to *marA*, isolated previously by selection with tetracycline and chloramphenicol, and which also carried nalidixic acid resistance (Cohen *et al* 1989). *MarA* resistance was linked to decreased drug permeability as a consequence of reduced OMPF expression and altered LPS. *NfxB* also appears to have a functional relationship with *marA*, as inactivation of *marA* by transposon insertion caused the loss of the ability to recover the *nfxB* mutation. It is thought that the *marA*$^+$ genes encode factors which negatively regulate OMPF expression and, therefore, mutations in *marA* may increase the expression of this factor.

Decreases in OMPF proteins have been implicated in fleroxacin resistance (Chapman *et al* 1989), norfloxacin resistance (Hirai *et al* 1986b) and ciprofloxacin resistance (Bedard *et al* 1989) in *E.coli*. The *norB* mutation results in low level resistance to quinolones, cefoxitin, tetracycline and chloramphenicol (Hirai *et al* 1986b). The *norC* mutation encodes hypersusceptibility to hydrophobic quinolones, such as nalidixic acid, and results in decreased OMPF expression coupled with LPS changes. Resistance brought

about by outer membrane protein mutations is thought to have less effect on nalidixic acid than modern 4-quinolones, as nalidixic acid is more hydrophobic and may, therefore, permeate into the bacterial cell through the lipid bilayer (Hirai *et al* 1986a). Other bacteria showing resistance to quinolones because of decreased expression of an OMPF protein include *Enterobacter cloacae,* where a decrease in OMPF and an increase in OMPC resulted in low level resistance to sparfloxacin, fleroxacin, temafloxacin and ciprofloxacin.

Reports in *Klebsiella* and *Serratia* species also implicate a reduction in outer membrane proteins in quinolone resistance (Gutmann *et al* 1985). In this report, however, resistance caused by altered permeability was insufficient to account for the total resistance. On investigating ofloxacin resistance in *Proteus vulgaris* low level resistance was linked to reduced expression of a 37kD porin protein which was thought to be analogous to OMPF in *E.coli* (Ishii *et al* 1991). Chapman *et al* (1989) suggested that as fleroxacin was less affected by OMPF deficiency than ciprofloxacin, there might be other routes of quinolone permeation into the cell. Studies on non-porin entry suggest that drugs such as ciprofloxacin, norfloxacin and enoxacin can utilise non-porin pathways, but that these are not the preferred routes (Bryan and Bedard 1991).

1.8.1.2 ALTERATIONS IN THE TARGET ENZYME.

The second chromosomally-mediated quinolone resistance is brought about by alterations in the bacterial DNA gyrase, and was originally associated with high levels of quinolone resistance, although experiments by Yoshida and co-workers (1988) have indicated that mutations in the *gyrA* gene of *E.coli* can cause various levels of resistance. Mutations in DNA gyrase have been reported in many bacterial species including *E.coli* (Hirai *et al* 1986b; Cullen *et al* 1989), *S.aureus* (Tanaka *et al* 1991), *P.aeruginosa* (Inoue *et al* 1987; Robillard and Scarpa 1988), *K.pneumoniae* (Piddock and Zhu 1991), *Providencia stuartii* (Heisig and Wiedemann 1991), *Enterococcus*

faecalis (Nakanishi *et al* 1991a), *Campylobacter jejuni* (Gootz and Martin 1991), *Serratia marcescens* (Fujimaki *et al* 1989), *S.epidermidis* (Sreedharan *et al* 1991), *E.cloacae* (Yamashita *et al* 1986), *N.gonorrhoeae* (Power *et al* 1992), *C.freundii* (Aoyama *et al* 1988b), *B.subtilis* (Lampe and Bott 1984) and *Proteus mirabilis* (Power *et al* 1992). A gyrase mutation coupled with a decrease in drug accumulation has also been implicated in the resistance of clinical strains of *Streptococcus pneumoniae* to 4-quinolones (Jin and Piddock 1993). The existence of DNA gyrase mutations in both Gram-negative and Gram-positive bacteria implicates them as the major resistance determining mechanism to 4-quinolones.

Alterations in the DNA gyrase have been documented in both the *gyrA* and *gyrB* genes, although the majority of mutations are found in the *gyrA* gene, indicating that it has a major part to play in quinolone resistance. The resistance genes in *E.coli* encoding *gyrA* resistance include *nalA, nfxA, norA, ofxA* and *cfxA* (Table 1.2) (Yamagishi *et al* 1981; 1986). Studies of *gyrA* resistance were further aided by the determination of the nucleotide sequences for two nalidixic acid-resistant and two pipemidic acid-resistant *E.coli* strains (Yoshida *et al* 1988). In each mutation a single base pair was altered. The four documented mutations were all located in the N-terminal sequence of *gyrA*, in the hydrophilic regions of the peptide, which suggests that they are situated near the surface of the α subunit. The mutations occur at amino acid serine 83, close to the codon tyrosine 122, which is the site of DNA attachment to the α subunit when DNA gyrase has cleaved the DNA in the presence of the quinolone antimicrobial agents (Horowitz and Wang 1987).

GENOTYPE	LINK	LOCATION (mins)	MECHANISM	PHENOTYPE
gyrA	cfxA, nfxA norA, ofxA, nalA	48	Alteration in α subunit	Resistance to quinolones only.
gyrB	nalC (nal31)	82	Alteration in β subunit	Hypersusceptible to new quinolones.
	nalD (nal24)	89	Alteration in β subunit and altered LPS.	Low level resistance to new quinolones.

Table 1.2. Mutations in the DNA Gyrase of *Escherichia coli.*

Yamagishi and colleagues (1981; 1986) identified two mutations in *E.coli, nalC* and *nalD,* when selecting with nalidixic acid. The *nalD* mutation was found to increase resistance to the new 4-quinolones, particularly those with piperazinyl substitutions, whilst *nalC* mutations appeared to determine an increased susceptibility to new quinolones. The mutations encoded single amino acid changes which altered protein charge in opposite directions, suggesting that charge may be related to drug-enzyme interaction.

There have been several mutation sites documented for *E.coli* (Table 1.3). All *gyrA* mutations were shown to fall between amino acids 67 and 106, and this has been termed the 'quinolone resistance-determining region' (Yoshida *et al* 1990a). The high-level resistance mutations have shown a single nucleotide change resulting in the alteration of Ser83 to leucine or tryptophan (Cullen *et al* 1989). Other mutations may occur in concert with a mutation at Ser83, or in combination with a mutation at Asp87. Low-level resistance-determining mutations were not isolated singularly, and always appeared in combination with another mutation. In all cases, experiments have established an alteration in Ser83 as the most common *gyrA* mutation, and that a mutation here is sufficient to confer high levels of quinolone resistance (Cullen *et al* 1989).

SUBUNIT	MUTATION (amino acid no.)	QUINOLONE RESISTANCE	REFERENCE
gyrA	Ala (67) to Ser	low level	Yoshida *et al* 1988
	Gly (81) to Cys	medium level	Yoshida *et al* 1990a
	Ser (83) to Ala	low level	Hallett and Maxwell 1991
	Ser (83) to Leu	high level	Yoshida *et al* 1990a
	Ser (83) to Trp	high level	Yoshida *et al* 1988
	Ala (84) to Pro	medium level	Yoshida *et al* 1988
	Asp (87) to Gly	high level	Heisig *et al* 1993
	Asp (87) to Asn	medium/high level	Yoshida *et al* 1990a
	Asp (87) to Val	low level	Oram and Fisher 1991
	Gln (106) to Arg	low level	Hallett and Maxwell 1991
	Gln (106) to His	low level	Yoshida *et al* 1990a
gyrB	Asp (426) to Asn	low/medium level	Yamagishi *et al* 1986
	Lys (447) to Glu	low level	Yamagishi *et al* 1986

Table 1.3. Quinolone-Resistant Mutations of *Escherichia coli* DNA Gyrase.

'Silent mutations' have been documented in *E.coli* (Oram and Fisher 1991), which appear to occur outside the quinolone resistance-determining region, and many strains with Ser83 mutations have been shown to carry the same silent mutations. Cullen and colleagues (1989) have also found silent mutations occurring outside the quinolone resistance-determining region which encode neutral changes of Asp678 to glutamine and Ala828 to serine. Neither of these mutations contributed to the resistant phenotype. Other bacteria exhibit similar resistance patterns to *E.coli* (Table 1.4). In *S.aureus gyrA* and *gyrB* genes were shown to have 66% homology with their *E.coli* and 76% homology with their *B.subtilis* counterparts (Hopewell *et al* 1990). Mutations in the *S.aureus* gyrase have been investigated, and amino acid changes which are analogous to those found in *E.coli* have been reported. Sreedharan and co-workers (1991) discovered cytosine to thymine transitions in *S.aureus* which conveyed a serine to leucine change at amino acid 84, and/or a Ser85 to proline codon change.These are analogous to changes at amino acids 83 and 84 in *E.coli.* They also discovered a novel Ser84 to Phe mutation in *S.epidermidis.*

These documented changes have been verified by Fasching and colleagues (1991) in clinical isolates of MRSA with oligonucleotide probes, and by Goswitz *et al* (1992) by Polymerisation Chain Reaction (PCR) and direct DNA sequencing. Goswitz and colleagues (1992) reported serine to alanine mutations at amino acid 84 and glutamine to lysine at amino acid 88, with all the quinolone-resistant strains possessing at least one mutation between these amino acids.

Recent investigations by Wang and colleagues (1993) have identified amino acid changes in the *gyrA* gene of *C.jejuni*. The *gyrA* sequence of *C.jejuni* shows 50% identity with the sequences of other documented *gyrA* sequences, with the quinolone resistance-determining regions being virtually identical. The mutations found in *C.jejuni* occurred at Thr86, Asp90 and Ala70 which are analogous to Ser83, Asp87 and Ala67 in *E.coli*. The mutations at Thr86 and Ala70 conferred high and medium levels of quinolone resistance respectively.

SPECIES	SUBUNIT	AMINO ACID	MUTATION	QUINOLONE RESISTANCE	REFERENCE
Staph. aureus	gyrA	84 (83)*	Ser to Leu	High level	Sreedharan *et al* 1990
		84 (83)	Ser to Ala	Medium level	Goswitz *et al* 1992
		85 (84)	Ser to Pro	Medium level	Sreedharan *et al* 1990
		88 (87)	Glu to Lys	Medium level	Goswitz *et al* 1992
Staph.epidermidis	gyrA	84 (83)	Ser to Phe	Medium level	Sreedharan *et al* 1991
Camp. jejuni	gyrA	70 (67)	Ala to Thr	Low level	Wang *et al* 1993
		86 (83)	Thr to Ile	High level	Wang *et al* 1993
		90 (87)	Asp to Asn	Medium/low	Wang *et al* 1993
Haloferax	gyrB	82	Asp to Gly	-	Holmes and Dyall-Smith 1991
		122	Ser to Thr	-	Holmes and Dyall-Smith 1991
		137	Arg to His	-	Holmes and Dyall-Smith 1991
Mycobacterium tuberculosis	gyrA	90 (83)	Ala to Val	High level	Takiff *et al* 1993

( )* Equivalent amino acid position in *Escherichia coli.*

Table 1.4. Quinolone-Resistant Mutations of Other Bacterial DNA Gyrases.

Although *gyrA* mutations occur more frequently, *gyrB* mutations in *E.coli* may affect quinolone resistance. *GyrB* mutations have been implicated in low-level quinolone resistance (Inoue *et al* 1978) and their relevance to overall quinolone resistance has been questioned. It has been suggested that the peripheral parts of the 4-quinolone molecule may react with the β subunit of *E.coli* DNA gyrase, and so mutations affecting this may confer low level resistance. An alternative suggestion is that alterations in the β subunit may act indirectly by altering the conformation of the α subunit, and through this the quinolone binding site (Reece and Maxwell 1991). Recent work by Yoshida and colleagues (1993) has established that the β subunit does have a significant role to play in determining quinolone resistance.

The *nal31* mutation (Yamagishi *et al* 1986), also documented as the *nalC* mutation (Inoue *et al* 1978), and the *nal24* mutation (Yamagishi *et al* 1986) are both point mutations which alter amino acids 436 and 427 in the β subunit respectively. This region has been proposed to be the region involved in the binding of the β subunit to the α subunit (Cozzarelli 1980). The *nal24* mutation involves an asparagine to aspartic acid substitution, and the *nal31* one from lysine to glutamic acid. As in the *gyrA* mutations, these substitutions involve an alteration in the charge of the β subunit. The *nal24* mutation decreases the negative charge of the protein at that point, and the *nal31* increases the negative charge.

Investigations into novobiocin resistance in *Haloferax* by Holmes and Dyall-Smith (1991) found similar *gyrB* mutations to those reported in *E.coli*. On sequencing the novobiocin resistance gene the documented amino acid changes were Asp82 to glycine, Ser122 to threonine, and Arg137 to histidine. On further investigation it remained unclear which amino acid changes were responsible for the resistant phenotype and which, if any, were silent mutations.

The effect of both *gyrA* and *gyrB* mutations seems to involve an alteration of the charge or hydrophobicity in the relevant amino acid. Two hypotheses have been proposed to explain how quinolone resistance is brought about by these alterations. The first suggests that mutations involving amino acid changes might alter the structure of the enzyme to interfere with quinolone activity (Goswitz *et al* 1992). The second, and more widely accepted hypothesis, is that an alteration in charge either prevents or facilitates the binding of quinolones (Yoshida *et al* 1991; Goswitz *et al* 1992). This theory is further proved by the observations of Smith (1984b), where the *nal31* mutation only conferred resistance to acidic 4-quinolones lacking a position 7 piperazine substituent; but at the same time rendered bacteria hypersensitive to the amphoteric quinolones possessing a position 7 piperazine substituent, which is positively charged. This anomaly is thought to occur as amphoteric quinolones have a positive grouping in an ideal position to interact with the β subunit at the mutation site (Yoshida *et al* 1991).

1.8.1.3 ACTIVE EFFLUX MECHANISM.

Until recently, tetracycline was thought to be the only antibiotic which was actively excreted from the bacterial cell (McMurry *et al* 1980). Examples of efflux based resistance to hydrophilic quinolones, however, have been documented in clinical isolates of *S.aureus* (Nakanishi *et al* 1991b; Tanaka *et al* 1991), *P.vulgaris* (Ishii *et al* 1991) and *E.coli* (Hooper *et al* 1989; Kotera *et al* 1991).

In *S.aureus* it has been suggested that the gene *norA* might be linked to quinolone efflux from the cell. *NorA* encodes a hydrophobic membrane protein of approximately 43kD (Ubukata *et al* 1989) and is dominant over the wild-type quinolone-susceptible phenotype of *E.coli*, so is unlikely to involve gyrase mutations (Kaatz *et al* 1991). The membrane protein encoded by *norA* displays homology with other transport proteins known to be coupled to the electrochemical proton gradient (Yoshida *et al* 1990c). Another *norA* type gene, *norA1199,* was recently isolated by Kaatz and co-workers

(1993) and was established to be a multidrug efflux transporter, which transported hydrophilic quinolones faster than hydrophobic ones. *NorA1199* exhibits significant homology with tetracycline efflux proteins but is chromosomally-encoded, instead of plasmid-encoded like its tetracycline counterpart. *NorA1199* was reported to show a high degree of homology with the *B.subtilis Bmr* protein, which mediates the efflux of chloramphenicol and fluoroquinolones (Neyfakh *et al* 1993).

Efflux systems have been reported in the inner membrane of *E.coli* and are energy requiring and saturable. Efflux was identified by using everted inner membrane vesicles in which norfloxacin accumulation reflected drug efflux in a normal intact cell (Cohen *et al* 1988). Active efflux was demonstrated to be a resistance mechanism in *P.vulgaris* by the use of carbonylcyanide m-chloro-phenylhydrazone (CCCP) an energy inhibitor. Ishii *et al* (1991) determined that ofloxacin accumulation in *P.vulgaris* was forty-fold higher in the presence of CCCP than in its absence. CCCP was also used to demonstrate active efflux of hydrophilic quinolones in *S.aureus* (Nakanishi *et al* 1991b) and efflux of norfloxacin in *E.coli* (Hooper *et al* 1989).

In all these reports, active drug efflux has not been implicated as the sole mechanism of bacterial resistance to quinolones; indeed it seems to play a secondary role in quinolone resistance compared to permeability changes and DNA gyrase mutations. When coupled with other resistance mechanisms, however, active efflux of hydrophilic quinolones from the bacterial cell has contributed to highly resistant organisms, which if selected further could cause many problems clinically.

1.9 MECHANISMS OF QUINOLONE RESISTANCE IN *PSEUDOMONAS AERUGINOSA.*

P.aeruginosa has been shown to be highly resistant to quinolones both *in vivo* and *in vitro*, and this resistance is becoming increasingly important clinically. Quinolone resistance in *P.aeruginosa*, like other bacteria, is caused by permeability and gyrase mutations and drug efflux mechanisms, enabling the resistant bacteria to be broadly categorised :-

Type 1 : Resistant to all quinolones but susceptible to unrelated antibiotics, mediated by gyrase mutations.

Type 2 : Resistant to quinolones, carbenicillin, novobiocin and chloramphenicol, caused by decreased drug penetration.

Type 3 : Resistant to quinolones and chloramphenicol but hypersusceptible to gentamicin and carbenicillin, probably via decreased drug transport (Yoshida *et al* 1990b).

The categories were also classified by their ability to be transformed by the wild-type *E.coli gyrA* gene. Type 1 isolates became quinolone-susceptible, whereas types 2 and 3 demonstrated no alteration in resistance. This classification, however, does not take into consideration the ability of *P.aeruginosa* to exhibit more than one quinolone resistance mechanism (Reece and Maxwell 1991).

1.9.1 Permeability Mutations.

The quinolone resistance mechanism in *P.aeruginosa* that has been most widely documented is that produced by permeability mutations. Several porin proteins have been implicated in the resistance to antimicrobial agents in *P.aeruginosa.* These include *OprE* (OMPE), *OprD* ($OMPD_2$), *OprB* ($OMPD_1$) and OMPC (Hancock *et al* 1990). Resistance to quinolones via permeability mutations in *P.aeruginosa* may also result in cross resistance to unrelated antimicrobial agents, for example imipenem (Rådberg *et al*

1990) and β–lactam antibiotics (Rella and Haas 1982; Fukuda *et al* 1990). The documented alterations in outer membrane proteins in *P.aeruginosa* are similar to those seen in *E.coli* in that they confer less resistance to nalidixic acid than to the fluorinated quinolones (Lewin *et al* 1990). The mutations differ from those reported in *E.coli,* however, in that several outer membrane proteins are implicated and not just OMPF (Table 1.5).

GENOTYPE	MECHANISM	CHARACTERISTICS	REFERENCE
nalB	-	High level quinolone resistance, low level nalidixic acid resistance	Rella and Haas 1982
cfxB	Extra 51kD protein	High level quinolone resistance, cross resistance to tet, chlor, nov and cab.	Robillard and Scarpa 1988
Qr1, Qr2	reduction OmpG and 40kD protein	Cross resistance to β-lactams, tet. and chloramphenicol.	Chamberland *et al* 1989
nfxB	New 54kD protein	Hypersusceptibility to β-lactams and aminoglycosides.	Hirai *et al* 1987
nfxC	Increased expression 50kD protein	Cross resistance to imipenem and chlor and hypersusceptibility to β-lactams and aminoglycosides.	Fukuda *et al* 1990

tet - tetracycline, chlor - chloramphenicol, nov - novobiocin, cab - carbenicillin.

Table 1.5. Permeability Mutations in *Pseudomonas aeruginosa.*

Resistance to quinolones has been linked with the loss of OMPF in *P.aeruginosa* and conferred ureidopenicillin cross resistance (Piddock *et al* 1987), but most resistance has been associated with other membrane proteins. Resistance linked to the *cfxB* and *nalB* mutations was shown to be unrelated to OMPF, and exhibited cross resistance to carbenicillin. The *nalB* mutation, later termed *cfxB*, conferred high level resistance to ciprofloxacin, ofloxacin, norfloxacin and enoxacin, on a par with gyrase mutations, but

only low level resistance to nalidixic acid (Rella and Haas 1982; Robillard and Scarpa 1988). The *cfxB* mutation encoded an additional 51kD protein and exhibited cross resistance to chloramphenicol, tetracycline and novobiocin as well as carbenicillin.

Other investigators have implicated the loss of, or reduced expression of, a 31-32kD outer membrane protein in low-level quinolone resistance in clinical isolates of *P.aeruginosa* (Daikos *et al* 1988; Kaatz and Seo 1988). This alteration in a 31-32kD protein was unlike the *cfxB* mutation as it was unstable, reverting back to ciprofloxacin sensitivity after serial passaging, and it conferred no cross resistance to unrelated antimicrobial agents.

Chamberland and colleagues (1989) reported low level quinolone resistance in two norfloxacin-resistant mutants of *P.aeruginosa* , termed Qr1 and Qr2. The altered permeability of these bacteria did not reside in major rearrangements of outer membrane proteins, or a modification of OMPF. Instead it was associated with a reduction in OMPG and an antigenically related 40kD outer membrane protein. The function of OMPG could not be established, and the relationship between the two proteins was unclear, but the mutant bacteria also exhibited cross resistance to some β-lactams, chloramphenicol and tetracycline.

Quinolone resistance in *P.aeruginosa* is not solely associated with decreased expression of outer membrane proteins, but has also been reported to involve the increase of certain proteins. Hirai and colleagues (1987) demonstrated that norfloxacin resistance in *P.aeruginosa*, designated *nfxB*, was linked to the appearance of a 54kD protein by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The *nfxB* mutation was unlike any other, as instead of conferring cross resistance to β-lactams and aminoglycosides like *cfxB*, it conferred hypersusceptibility to these drugs.

Furthermore, the *nfxB* mutation was not linked to chloramphenicol and tetracycline susceptibility or resistance.

The presence of a 54kD protein conferring quinolone resistance was also documented by Legakis *et al* (1989) when utilising ciprofloxacin selection. Resistance involved increased expression of this protein, however, rather than the appearance of a new protein. The mutation was established to be different from *nfxB* as it encoded cross resistance to β-lactams and aminoglycosides. Fukuda and co-workers (1990) have implicated an increase in a 50kD protein in quinolone resistance, as mutations designated *nfxC* produced large amounts of this protein on SDS-PAGE. Overproduction of this 50kD protein was linked with resistance to imipenem and chloramphenicol and hypersusceptibility to β-lactams and aminoglycosides. The *nfxC* mutants also exhibited reduced expression of OMPG, but unlike the findings of Chamberland and colleagues (1989), no alterations in a 40kD protein were documented. Fukuda *et al* (1990) also reported that *nfxC* mutants were cross resistant to imipenem and that this was linked to the decreased expression of a 46kD protein.

Quinolone and imipenem cross resistance has been reported in clinical isolates of *P.aeruginosa* (Rådberg *et al* 1990), and has been associated with reduced expression of protein D_2, a 47kD outer membrane protein (Rohner *et al* 1992). Resistance caused by decreased expression of protein D_2 was linked with increases in the amount of cell surface LPS, although the LPS alterations were thought to exert a minimal effect on resistance. Furthermore, alterations in LPS coupled with outer membrane protein changes have been implicated clinically in ciprofloxacin-resistant *P.aeruginosa* strains from cystic fibrosis patients (Diver *et al* 1991). Legakis and co-workers (1989) also demonstrated that permeability mutations were linked with truncated LPS in *P.aeruginosa*, and Michéa-Hamzehpour and colleagues (1991) suggested that an increase in LPS expression in ciprofloxacin- and norfloxacin-resistant *P.aeruginosa*

might produce a permeability barrier which restricted the penetration of these hydrophilic quinolones into the bacterial cell.

To summarise, permeability mutations in *P.aeruginosa* , unlike *E.coli*, are not solely associated with the decreased expression of one protein, but instead are associated with either alterations, reduced expression or loss of several proteins, or the increased expression and appearance of new proteins. They are also invariably coupled with alterations in bacterial LPS expression or with DNA gyrase mutations.

1.9.2 DNA Gyrase Mutations.

There have been reports of mutations in both the *P.aeruginosa* α and β subunits of DNA gyrase. The main documented mutations in *gyrA* are *nalA* (Hirai *et al* 1987; Inoue *et al* 1987), *cfxA* (Robillard and Scarpa 1988), and *nfxA* (Fukuda *et al* 1990), and one in *gyrB* (Yoshida *et al* 1990b). All of the mutations were identified by the transformation of a sensitive *E.coli gyrA* gene into resistant isolates of *P.aeruginosa*, which revert to quinolone susceptibility if a *gyrA* mutation is present.

Inoue and co-workers (1987) separated the subunits of DNA gyrase and showed via inhibition tests on reconstituted gyrases, containing combinations of α and β subunits, that resistant strains had alterations in the α subunit. These mutations were subsequently found to map on the *nalA* gene. Inoue and colleagues (1987) concluded that the product of the *nalA* gene was the gyrase α subunit protein, and that mutations in this protein were responsible for high levels of quinolone resistance in *P.aeruginosa*. Later Robillard and Scarpa (1988) isolated *cfxA* mutations in laboratory mutants of *P.aeruginosa*, selected with ciprofloxacin, and via the isolation of DNA gyrase and inhibition studies demonstrated that the mutations conferred high level quinolone resistance, and that they too mapped in the *nalA* region of the *P.aeruginosa* genome.

Transformation experiments by Yoshida and colleagues (1990b) identified a *P.aeruginosa* isolate as being *gyrB* resistant when a plasmid carrying the *E.coli* wild type *gyrB* conferred an eight-fold susceptibility to enoxacin on the strain. Yoshida and co-workers (1990b) also reported 12 isolates carrying *gyrA* mutations. Transformation methods have also been employed by Heisig and Wiedemann (1991) and Piddock and co-workers (1992) to show *gyrA* mutations in clinical isolates of *P.aeruginosa*. So far, the *gyrA* sequence of *P.aeruginosa* has not been published, therefore specific *gyrA* mutations in this organism have not been identified further.

1.9.3 Efflux Mechanisms.

Both Bryan and colleagues (1989) and Bedard and co-workers (1989) have reported increases in whole cell accumulation of norfloxacin, ciprofloxacin and enoxacin in *P.aeruginosa* isolates treated with CCCP. They also reported that sodium azide, sodium fluoride, sodium arsenate and EDTA are able to increase ciprofloxacin accumulation in *P.aeruginosa* mutants as well as CCCP and DNP. This would indicate the presence of an active efflux mechanism in *P.aeruginosa*, although this has been established to have a minor effect on quinolone resistance compared to gyrase mutations or alterations in drug permeability.

1.10 AIMS OF THIS THESIS.

This thesis investigated the mechanisms of resistance of *P.aeruginosa* to the 4-quinolones. The aims of this thesis were:-

1) To investigate the nature and the frequency of outer membrane protein mutations in clinical and laboratory isolates of *P.aeruginosa*, and to determine the effects of these mutations upon quinolone resistance.

2) To determine the nature and frequency of DNA gyrase mutations in ciprofloxacin-resistant clinical isolates of *P.aeruginosa* by investigating their specific activities.

3) To investigate possible mutation sites in the DNA gyrase of clinical isolates of *P.aeruginosa*, and their frequency of occurrence.

4) To establish the relationship between MICs, OMP alterations and DNA gyrase mutations.

MATERIALS AND METHODS.

2.1 REAGENTS.

All chemicals and reagents were supplied by Sigma Chemical Co. Ltd. unless otherwise stated.

2.2 BACTERIAL STRAINS.

Forty clinical isolates were investigated in this thesis, and are listed in Tables 2.1 and 2.2. The bacterial isolates were stored at -70°C in a 10% skimmed milk solution (Oxoid). Strains were always subcultured from the stock and not passaged. All strains were pyocin-typed by the method of Govan *et al* (1983) to verify that they were the *P.aeruginosa* isolates stated.

STRAIN	PYOCIN TYPE	SOURCE	ISOLATED FROM:-
91-32	1/d (s-a)*	GRI	Blood culture on 21/3/91
91-33	1/d (s-a)	GRI	Blood culture on 28/3/91
91-40	1/d (s-a)	GRI	Blood culture on 6/4/91
91-41	1/d (s-a)	GRI	Blood culture on 5/4/91
91-42	1/d (s-a)	GRI	Blood culture on 8/4/91
91-44	1/d (s-a)	GRI	Blood culture on 9/4/91
91-46	1/d (s-a)	GRI	MIC plate of 91-40
91-76	10/d	GRI	Blood culture 11/4/91
91-28	44/m	GRI	Patient II Blood culture on 20/3/91
91-36	ut/m	GRI	Patient II Blood culture on 28/3/91
90-62	1/c (s-5)	GRI	Patient III Blood culture on 27/7/90
90-67	1/c (s-5)	GRI	Patient III Blood culture on 20/8/90

ut - untypable strains * (s-) sensitivity shown to reagents named

Table 2.1. Origins of Paired Clinical Strains.

STRAIN	PYOCIN TYPE	SOURCE
PAO1	-	Public Health Laboratory Service, (PHLS) Colindale.
U423	76/ut (s-7,a,d,e)*	Royal Infirmary of Edinburgh (RIE)
C1	ut/v	RIE
C2	10/h (s-b)	RIE
C32	105/w	RIE
C48	10/c (s-3,6,7)	RIE
C48s	10/c (s-3,6,7)	This laboratory
C49	1/a (s-4,6)	RIE
3	ut/a (s-b,d)	Royal Infirmary of Cardiff (RIC)
48	10/c (s-3,6,7)	RIE
52	ut/k	RIE
135	1/d	RIE
271	10/a (s-7)	RIC
273	ut/p (s-5)	RIC
274	48/ut (s-1,2,5,6,7,a,d,e)	RIC
4124	10/ut (s-a)	Leicester, (PHLS Colindale)
4125	10/ut (s-a)	Leicester, (PHLS Colindale)
4131	10/h (s-b)	Shrewsbury, (PHLS Colindale)
4133	10/h (s-b)	Shrewsbury, (PHLS Colindale)
4149	10/c (s-3,6,7)	PHLS Colindale
4158	3/e (s-3)	Glasgow Royal Infirmary (GRI)
4161	3/e (s-5)	GRI
4269	1/a (s-4,6)	Exeter, (PHLS Colindale)
4352	9/h	France, (PHLS Colindale)
4374	9/ut	France, (PHLS Colindale)
4375	10/c (s-3,6)	PHLS Colindale
4495	ut/k	Isle of Wight, (PHLS Colindale)
4532	5/ut	PHLS Colindale

* ut - untypable strains (s-) sensitivity shown to reagents named

Table 2.2. Origin of Unpaired Clinical Strains.

2.3 ANTIBIOTICS.

Antimicrobial agents were supplied as sterile powders from the manufacturers. The 4-quinolones ciprofloxacin, ofloxacin (Tarivid®, Roussel Laboratories Ltd.) and norfloxacin (Noroxin®, Merck, Sharp and Dohme) were weighed aseptically, dissolved in sterile 0.1M sodium hydroxide solution (NaOH) and diluted to the required concentration with sterile distilled water. Imipenem (Primaxin®, MSD.), ceftazidime (Fortum®, Glaxo Laboratories Ltd.), cefotaxime (Claforan®, Roussel), gentamicin, carbenicillin (Pyogen®, SmithKline Beecham), tetracycline, ampicillin (SmithKline Beecham) and kanamycin were dissolved in pyrogen-free water (Milli-Q, Millipore). Trimethoprim (Wellcome Foundation Ltd.) was dissolved in lactic acid (Fisons) and sterile distilled water. All drug solutions were prepared fresh when needed. Drugs were stored as dry powders in darkness at 4°C.

2.4 MEDIA.

All growth media, both broth and agar, were sterilised by autoclaving at 121°C at 15 pounds/square inch for 15 minutes.

2.4.1 Complex Media.

The following complex media were used; Isosensitest Broth (IST, CM473) and IST agar (CM471), nutrient agar (CM3), and nutrient broth no. 2 (CM67) supplied by Oxoid. PIA was supplied by Difco.

2.4.2 Davis-Mingioli Minimal Salts Media.

Double strength and single strength minimal salts media were prepared as described by Davis and Mingioli (1950), the ingredients of which are shown in Table 2.3. Davis and Mingioli (DM) agar plates were prepared with 50ml of double-strength DM media supplemented with the appropriate amount of the required amino acid stock solutions, to achieve the final concentrations as shown in Table 2.4. Appropriate amounts of

selecting antibiotics were then added, as was a 20% D-glucose solution to give a final concentration of 0.28%. The volume was then made up to 60ml with sterile distilled water. This solution was added to 40ml of sterile molten Bacteriological Agar No. 1 (1g agar / 40ml distilled water, Oxoid) and the plates poured immediately.

INGREDIENTS	QUANTITY IN 1 LITRE (g)
K_2HPO_4	14.0
KH_2PO_4	6.0
Tri-sodium-citrate	1.0
$MgSO_4.7H_2O$	0.2
$(NH_4)_2SO_4$	2.0

Table 2.3. Ingredients of Double Strength DM Media.

SOLUTION	STRENGTH OF STOCK SOLUTION	FINAL CONCENTRATION IN DM AGAR
L-Histidine (BDH)	5g/l	50mg/l
L-Methionine (BDH)	5g/l	50mg/l
L-Proline (BDH)	5g/l	50mg/l
L-Tryptophan (BDH)	2g/l	50mg/l

Table 2.4. Concentrations of Amino Acid Supplements.

All amino acid solutions were sterilised by steaming for 30 minutes and added aseptically.

2.4.3 SOC Broth.

The constituents of one litre of SOC broth were as follows:-

20g Bacto tryptone (Difco); 5g Bacto yeast extract (Difco); 10mM NaCl; 2.5mM KCl; 10mM $MgCl_2$; 10mM $MgSO_4$; and 20mM glucose. The pH was adjusted to 7.0 with NaOH.

2.4.4 Hepes/Glycerol (HGEB) Broth.

The constituents of one litre of HGEB broth were :- 1mM Hepes solution (pH 7.0) and 10% w/v glycerol.

2.4.5 Luria-Bertani (LB) Broth

The constituents of one litre of L broth were, 10g Bacto peptone (Difco); 5g Bacto yeast extract (Difco) and 5g Sodium Chloride.

2.4.6 *Pseudomonas* (P) Broth.

The constituents for a litre of P broth are shown in Table 2.5. The broth was prepared by autoclaving the magnesium sulphate and the glucose separately, and then adding them to the remaining constituents, just prior to the inoculation of the starter cultures.

CONSTITUENT	AMOUNT g/l
Bacto peptone (Difco)	10
Bacto yeast extract (Difco)	2
$Na_2HPO_4.2H_2O$	8
K_2HPO_4	2
Ammonium sulphate	1.2
D-Glucose	10
$MgSO_4.7H_2O$	4.1

Table 2.5. The Constituents of P Broth.

2.5 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS (MICs)

MICs were determined for 34 quinolone-resistant and 7 quinolone-sensitive clinical isolates of *Pseudomonas aeruginosa,* and one reference strain, PAO1. Strains with MICs greater than the breakpoints determined by the Working Party of the British Society of Antimicrobial Chemotherapy (BSAC 1991) were deemed resistant to the antimicrobial concerned. The break points were as follows :- cefotaxime, 1mg/ l; ceftazidime 2mg/ l; imipenem 4mg/ l; ciprofloxacin 4mg/ l; ofloxacin 8mg/ l; norfloxacin 4mg/ l; tetracycline 1mg/ l; chloramphenicol, 8mg/ l; trimethoprim 0.5mg/ l; ampicillin, 8mg/ l; gentamicin 1mg/ l and carbenicillin 32mg/ l.

MICs were determined by an agar dilution technique on both IST agar and PIA by following a doubling dilution schedule unless otherwise stated. The antibiotic plates were inoculated with a multipoint inoculator (Denley) delivering approximately 10^5 colony forming units (cfu) per spot, and were incubated aerobically at 37°C unless otherwise stated. The MIC was defined as the lowest concentration of antibiotic at which no visible growth of bacteria was observed. The technique was validated by type strains of *Pseudomonas* (NCTC 10662), *Escherichia coli* (NCTC 10418) and *Staphylococcus aureus* (NCTC 6571), and consistency of results was ensured by including PAO1 as a standard on every plate.

2.6 MECHANISMS OF QUINOLONE RESISTANCE.

2.6.1 Stability of Quinolone Resistance in Clinical Isolates of *Pseudomonas aeruginosa.*

MICs of ciprofloxacin were determined on IST agar for 20 ciprofloxacin-resistant clinical isolates. Strains were streaked out for single colonies on drug-free PIA and incubated aerobically overnight at 37°C. A single colony was then passaged onto new PIA plates and treated as before. Strains were passaged daily for 15 days, and their MICs determined initially before passaging, and subsequently every five days.

2.6.2 Production of 4-Quinolone Resistant Mutant Isolates.

To produce spontaneous mutant bacteria, universals containing 10ml of drug-free nutrient broth were inoculated with a single colony of *P.aeruginosa* and incubated aerobically overnight at 37°C. The resulting cultures were harvested by centrifugation at 3000 rpm (Sorvall RT 6000D, DuPont) for 20 minutes and the resultant pellet resuspended in 1ml fresh nutrient broth, to give cell concentrations in excess of 10^{10} cfu per ml. Aliquots (0.1ml) of undiluted, 10^{-2}, 10^{-4} and 10^{-6} dilutions were spread onto IST agar plates containing either ciprofloxacin or ofloxacin at 2, 5 and 10 times the respective MICs. The plates were incubated at 37°C for 24 or 48 hours. Resistant colonies were subcultured onto PIA plates containing antibiotic at the same concentration to verify resistance, and colonies pyocin typed to link them to the parent strain.

2.7 ALTERATIONS IN OUTER MEMBRANE PROTEINS (OMPs).

2.7.1 Preparation of OMPs.

All 22 quinolone-resistant isolates, 5 sensitive clinical isolates, 14 paired isolates and PAO1 were investigated for OMP alterations. The quinolone-resistant mutants obtained from the mutation experiments were also examined for OMP alterations. OMPs were prepared from two 250ml nutrient broth cultures grown shaking, aerobically, overnight at 37°C. Cells were harvested by centrifugation at 6000rpm (GS3 rotor, Sorvall RC-5B, DuPont) for 15 minutes and lysed by sonication at 8μm for four 30 second bursts, separated by 30 second cooling periods, with an ultrasonic disruptor (MSE Soniprep 150, MSE Instruments).

The lysate was then centrifuged three times at 12,000rpm (SA600 rotor, Sorvall RC-5B DuPont) for 15 minutes to remove whole cells, and the inner membranes solubilised by the addition of lauryl sarcosinate (sarcosyl) to a concentration of 0.7%. The outer membranes were isolated by ultracentrifugation at 100,000g (TFT 70.38 rotor, Sorvall OTD65-B, DuPont) for 1 hour at 4°C. The membranes were washed with 5ml pyrogen-free water (Milli-Q, Millipore) and harvested by centrifugation at 100,000g for 1 hour at 4°C. The precipitated membranes were finally resuspended in 1ml Milli-Q water and stored at -20°C.

2.7.2 Analysis of Outer Membrane Proteins by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

OMPs were diluted in Milli-Q to a concentration of 1mg/ml. Protein concentration estimates in the OMP preparations were determined by the method of Waddell (1956). The samples were diluted in sterile distilled water and their absorbance was measured at wavelengths of 215 and 225nm on a Perkin-Elmer UV/Vis Lambda II Spectrophotometer. The protein concentration was determined by reference to a previously prepared standard curve.

The proteins were resolubilised prior to electrophoresis by boiling in a SDS sample buffer for 5 minutes. The OMPs were analysed either on a gel gradient of 10-15% polyacrylamide (Pharmacia LKB.) on the PhastSystem® (Pharmacia), or on a gel gradient of 12% polyacrylamide (BioRad) on the BioRad Mini Protean System®. A coomassie brilliant blue R staining technique was employed to examine the separated protein bands.

2.7.3 Examination of OMPs For Non-Covalent Association With Peptidoglycan.

The method of Darveau *et al* (1983) was employed to investigate whether the OMPs were non-covalently associated with peptidoglycan. Briefly, cell envelopes, isolated by the outer membrane protein technique, were solubilised in a buffer containing 2% (w/v) SDS, 10% (w/v) glycerol and 10mM Tris-HCl (pH 7.4) at 30°C for 30 minutes. Envelopes were isolated by centrifugation at 100,000g for 1 hour and resuspended in the same buffer containing 0.1M sodium chloride. The resulting extract was analysed by SDS-PAGE on the Mini Protean® system as described above.

2.8 ANALYSIS OF DNA GYRASE.

2.8.1 Preparation of the Novobiocin/Sepharose Column.

A novobiocin/sepharose column was prepared according to the method of Staudenbauer and Orr (1981), with modifications. Epoxy-activated Sepharose 6B (5g, Pharmacia) was swelled overnight at room temperature in 400ml Milli-Q. The water was removed by a vacuum pump, and the beads washed with 150ml of 0.2M sodium carbonate buffer (pH 9.5). The beads were then resuspended in 100ml 0.2M sodium carbonate buffer (pH 9.5) and transferred to a shaking water bath at 37°C. After an hour, 700mg of novobiocin in 0.2M sodium carbonate buffer was added, and the mixture incubated, shaking, overnight at 37°C.

Next, 100ml of 0.1M Tris-HCl buffer (pH 7.5) was added to the flask, and the mixture incubated for a further 4 hours, shaking at 37°C. The beads were washed with 200ml of 0.5M sodium chloride in 0.2M sodium carbonate buffer (pH 9.5) with a vacuum pump, and washed again with Milli-Q water. The beads were then washed with 200ml of 0.5M sodium chloride in 0.2M sodium acetate buffer (pH 4.5) and again with 200ml of Milli-Q. In the final step the beads were washed with 200ml TED buffer containing 10mM Tris-HCl (pH 7.5), 1mM ethylene diamine tetraacetic acid (EDTA) (pH 8.0) and 1mM dithiothreitol (DTT). The beads were resuspended in 50ml TED buffer and the column poured. The column was washed with 100ml TED buffer containing 5M urea, and then washed with TED buffer for 20-30 minutes, until the eluate was clear.

2.8.2 Preparation and Isolation of DNA Gyrase.

DNA gyrase was prepared according to the method of Inoue and colleagues (1987), with modifications. In brief, 10ml of starter culture was grown, shaking, at 37°C overnight and added to a litre of P broth. For each strain 4-6 litres of culture were grown shaking at 37°C for 4-6 hours in P broth. Cells were harvested by centrifugation at 6,500rpm for 20 minutes, washed in TED buffer, and resuspended in 100ml TED buffer. Cells were lysed chemically with 1.4ml of 200mM DTT, 5.5ml of 0.5M EDTA (pH 8.0) and 13.8ml of 1M potassium chloride (KCl). The solution was mixed gently and 5.5ml of 20mg/ml lysozyme and 10% (w/v) polyoxyethylene 20 cetyl ether (Brij 58) added. The mixture was then incubated, stirring, at 4°C for 2 hours, or until complete lysis of the cells had occurred.

The cell extract was isolated by centrifugation at 100,000g at 4°C for 1 hour, and the DNA precipitated by the addition of 2% (w/v) streptomycin sulphate. The mixture was centrifuged at 10,000rpm for 15 minutes and unwanted proteins were removed from the supernatant by 20% (w/v) ammonium sulphate precipitation at 4°C; with the floating precipitate being removed after centrifugation at 10,000g for 15 minutes. The crude enzyme was then isolated by 40% (w/v) ammonium sulphate precipitation, with the precipitated pellet being resuspended in 10ml TED. The crude enzyme was then dialysed overnight in TED buffer at 4°C.

Sterile dialysis tubing was prepared by boiling the tubing (visking size 8/32" Medicell International Ltd.) in one litre of distilled water containing 2ml of 0.5M EDTA and 20g of sodium bicarbonate. The tubing was boiled for 10 minutes in a water bath and washed four times in sterile distilled water. The dialysis tubing was then sterilised by autoclaving prior to use.

DNA gyrase was isolated on a novobiocin/sepharose column at 4°C. The column was equilibrated with TED buffer before the addition of the crude enzyme preparation, and then eluted with 0.1M KCl in TED buffer to remove any unwanted proteins and DNAses. The DNA gyrase was eluted with 5M urea in TED buffer and the resulting fractions dialysed in 1ml aliquots, twice against TED buffer at 4°C for two hours, and then against TED buffer with 50% (v/v) glycerol (Molecular Biology Reagent Grade, Sigma, TGED) overnight. The purified enzyme was stored in TGED buffer at -70°C for up to a month.

2.8.3 Relaxation of pBR322.

The relaxation of pBR322 (Northumbria Biologicals Ltd.) was carried out by mixing the reagents in Table 2.6. The reagents were mixed and incubated at 37°C for 2 hours. The same volume of 80% phenol, equilibrated in TED buffer, was added and the mixture vortexed and centrifuged at 13,000rpm in an MSE Micro Centaur for a minute. The lower phenol layer was discarded, and an equal volume of 1:1 phenol: chloroform added to the aqueous layer. The mixture was again vortexed and centrifuged, and the lower layer discarded. An equal volume of chloroform was added, vortexed and centrifuged, and the upper layer added to an equal volume of ether, previously equilibrated in TED buffer. The ether layer was then removed, and any remaining ether evaporated off in a 60°C water bath. The relaxed state of the pBR322 was confirmed by gel electrophoresis on 0.8% agarose (refer to Materials and Methods section 2.8.4). The relaxed pBR322 was stored at 4°C.

REAGENT	AMOUNT ADDED
1M Tris-HCl buffer (pH 7.5)	45µl
1M KCl	45µl
200mM magnesium chloride	45µl
50mM DTT	45µl
1mg/ml bovine serum albumin	45µl
2mM EDTA (pH 8.0)	120µl
330 mg/ml pBR322 (NBL)	534µl
Topoisomerase I (10 units/µl, Gibco-BRL)	21µl

Table 2.6. The Constituents of the pBR322 Relaxation Mixture.

2.8.4 Analysis of DNA Gyrase.

DNA gyrase activity was assayed by gel electrophoresis in a Horizon 20.25 horizontal gel tank (Gibco-BRL). Aliquots obtained from the novobiocin/sepharose column were assayed for their ability to supercoil relaxed pBR322.

To assay DNA gyrase activity, 8µl of the enzyme was added to 8µl of assay mix, containing relaxed pBR322 (Table 2.7) and 4µl Milli-Q. The reagents were incubated statically at 37°C for 1 hour, and then run on an 0.8% agarose gel in single strength TEAS buffer (pH 8.0). The constituents of 20x TEAS buffer were:- 121.1g Trizma base; 14.9g EDTA; 54.43g sodium acetate and 21.4g NaCl per litre.

INGREDIENTS	VOLUME (µl)
1M Tris-HCl (pH 7.5)	40
1M KCl	40
200mM MgCl2	40
50mM ATP	40
50mM DTT	40
1mg/ml BSA	40
50mM spermidine	40
1mg/ml t-RNA	40
Relaxed pBR322	90
Milli-Q water	90

Table 2.7. Constituents of the DNA Gyrase Assay Mix.

Agarose gels were prepared from TEAS buffer and were loaded with either the 20µl samples or a marker dye consisting of:- 2ml of 10 times TEAS buffer, 1mg of bromophenol blue, 12mg of sucrose, 0.8ml of 0.5M EDTA and Milli-Q to 20ml. The gel was run for fifteen minutes at 120mA, until the marker dye had left the wells. The gel was then submerged in buffer and run at 30mA overnight.

Gels were stained with 0.75mg/l ethidium bromide for 1-2 hours and visualised over a long wave ultraviolet light source (Ultra-violet Products Inc.). Aliquots producing supercoiling were stored at -70°C for up to a month.

2.8.5 Determination of IC_{50} Values.

Active DNA gyrase, as determined above, was assayed against ciprofloxacin, ofloxacin, norfloxacin and novobiocin by gel electrophoresis on 0.8% agarose, as described in section 2.8.4. Doubling dilutions of each drug were prepared in Milli-Q from 256mg/l to 0.06 mg/l for ciprofloxacin, ofloxacin and norfloxacin, and from 8mg/l to 0.06mg/l for novobiocin. The assay mixture consisted of 8µl active gyrase, 8µl relaxed pBR322 assay mix (Table 2.7, with no Milli-Q water and 180µl of relaxed pBR322) and 4µl of the relevant antibiotic concentration.

The reagents were incubated statically for 90 minutes at 37°C. Gels were run and stained as described previously and photographed under an ultraviolet light source. The concentration of antibiotic which inhibited 50% of the supercoiling of the relaxed pBR322 (IC_{50}) was calculated on a Chromoscan 3 densitometer (Joyce Loebl) with the Chromoscan 3 External Data Analysis VS. 1 software (Joyce Loebl).

2.9 ISOLATION AND PURIFICATION OF TOTAL GENOMIC DNA.

Total genomic DNA was prepared by the method of Zyskind and Bernstein (1989). Bacterial strains were grown in 4.5ml of nutrient broth overnight at 37°C and 1.2ml broth was spun down in a MSE Micro Centaur centrifuge at 13,000 rpm and resuspended in HTE buffer (50mM Tris-Cl pH 8.0; 20mM EDTA). Cells were lysed by 2% sarcosyl in HTE buffer and RNA removed by the addition of RNAse. The mixture was incubated for 15 minutes at 37°C, and lysis completed by the addition of pronase and incubating for one hour at 50°C. DNA was isolated by phenol/chloroform and ether extraction, and precipitated by the addition of 3M sodium acetate and isopropanol at -70°C for 30 minutes. The mixture was centrifuged at 13,000rpm for 10 minutes and the DNA pellet washed three times in 70% ethanol, dried and resuspended in TE buffer (10mM Tris-HCl (pH 8.0); 0.1mM EDTA).

2.9.1 Analysis of Genomic DNA.

Genomic DNA was analysed by the Polymerase Chain Reaction (PCR) technique. PCR was carried out based on the method of Disney and Dove (1991). The PCR was carried out with two specific primers taken from the quinolone resistance determining region of the *P.aeruginosa gyrA* gene. The sequence of the *P.aeruginosa gyrA* gene was kindly given by Dr L.E. Bryan and the primers supplied by the University of Edinburgh Department of Chemistry, King's Buildings. The sequences of the two primers were:-

5' GGA TCC CAT GCG CAC TTC GGT GTA 3' antisense

5' GGA TCC GTG CTT TAT GCC ATG AGC GAG 3' sense

Both primers incorporated a *BamH1* restriction site.

The PCR was carried out in a total reaction volume of 100µl (refer to Table 2.8) in 0.5ml polypropylene microcentrifuge tubes (Alpha Laboratories), with a Techne PHC-2 Dri-Block® Cycler (Cambridge Bioscience). The reaction mixtures were overlaid with two drops of mineral oil and subjected to the denaturation, annealing and extension cycles described in Table 2.9. After the PCR reaction was completed, samples were 'quenched' on ice to prevent any further reaction. The subsequent double-stranded DNA was extracted by ethanol precipitation and checked for purity by gel electrophoresis on 1.5% agarose as before.

INGREDIENTS	VOLUME	SUPPLIER
10x *Taq* reaction buffer (Mg^{2+} free)	10µl	Promega
25mM $MgCl_2$	10µl	Promega
4mM dNTP stock solution*	1µl	Boehringer Mannheim UK
P.aeruginosa gyrA sense primer	10 pmoles	-
P.aeruginosa gyrA antisense primer	10 pmoles	-
Genomic DNA preparation	2µl	-
Taq DNA polymerase	1 unit	Promega
Milli-Q water	to 100µl	Millipore

* 4mM dNTP stock solution: 1mM dTTP; 1mM dGTP; 1mM dCTP; 1mM dATP in 10mM Tris-Cl (pH 7.5).

Table 2.8 PCR Reaction Components.

SEGMENT	TEMP.	TIME	RAMP RATE	NUMBER OF CYCLES	FUNCTION
1	96°C	30 secs	48°C / min	1	DNA denaturation
	50°C	60 secs	10°C / min		Primer annealing
	70°C	90 secs	30°C / min		Primer extension
2	96°C	15 secs	48°C / min	24	DNA denaturation
	50°C	30 secs	10°C / min		Primer annealing
	70°C	90 secs	30°C / min		Primer extension
3	70°C	5 min	-	1	Final extension

Table 2.9 The PCR Heating Cycle Protocol.

2.10 DIRECT ANALYSIS OF DNA GYRASE *GYRA* MUTATIONS.

2.10.1 Restriction and Transformation Technique.

P.aeruginosa DNA was obtained by PCR techniques, as described in section 2.9.1. The PCR sequence was digested with a *BamH1* restriction enzyme (Gibco-BRL), as recommmended by the manufacturer, in a 'REact' buffer system supplied with the enzyme. The restricted DNA was then ligated into pUC18. Ligations were carried out in a 20µl reaction volume containing ligation mix (Gibco-BRL), digested vector, pUC18, and the restricted target DNA to which was added 0.1 unit of T_4 DNA ligase (Gibco-BRL). The ligation mixture was then incubated at 16°C overnight, after which the sample was transferred into the host cell.

MAX Efficiency DH5α™ Competent cells (Gibco-BRL) were stored at -70°C and thawed on ice. DNA, from the ligation mix, was added to 50µl aliquots of DH5α cells and gently mixed before incubation on ice for 30 minutes. The cells were subjected to a heat pulse for one minute, returned to the ice, and 800µl of SOC broth added. The cells were allowed to recover by incubation for one hour at 37°C, and then were plated onto IST agar plates containing 25µg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 50µg/ml of isopropyl β-D-thiogalactopyranoside (IPTG) and 100µl/ml ampicillin. To determine the transformation efficiency, a known concentration of pUC18 DNA was transformed, and the number of transformants calculated from the IST agar plates.

2.10.2 Direct Sequencing From PCR Products.

Single- and double-stranded DNA sequencing was carried out with the USB Sequenase® Version 2.0 DNA sequencing kit (Cambridge Bioscience), based on the chain-termination method of Sanger *et al* (1977). Reaction products were radiolabelled with [^{35}S] α-dATP label (Amersham International plc.) and separated by electrophoresis.

Electrophoresis was performed with the Sequi-Gen® Nucleic Acid Sequencing Cell (BioRad), in a denaturing polyacrylamide gel (8%T 5%C acrylamide /*bis*-acrylamide, 7M urea) under an electrolytic gradient. The Sequi-Gen Cell was cleaned and assembled according to the manufacturer's recommendations. The sequencing method employed was that of Winship (1989).

The denaturing polyacrylamide gel consisted of a plug solution containing 20ml 8%T 5%C acrylamide /*bis*acrylamide, 7M urea stock solution (Table 2.10), 140µl of 25% w/v ammonium persulphate and 50µl TEMED. The gel solution contained 55ml 8%T 5%C acrylamide /*bis*acrylamide, 7M urea stock solution, 220µl 25% w/v ammonium persulphate and 7µl TEMED.

INGREDIENTS	STOCK SOLUTION
Urea 10x TBE buffer 40%T 5%C acrylamide/N,N'-methylene-bis-acrylamide	7M single strength 8%T 5%C

Table 2.10. The Ingredients of the Denaturing Acrylamide/*bis*acrylamide Stock Solution.

The plug solution was allowed to polymerise for 10-15 minutes, and the electrophoresis gel allowed to polymerise for one hour. The inner plate chamber of the Sequi-Gen Cell was then filled with half strength TBE buffer (10x TBE stock solution contains: 0.89M Tris-borate (pH 8.3); 20mM EDTA), and the lower chamber was filled with 400ml of single strength TBE buffer. The gel was pre-run for 30-40 minutes at constant power (60W, 3000V and 50mA) to bring the apparatus to a running temperature of 50-55°C. Samples were loaded and the gel run for one hour. After this, 200ml of 3M sodium acetate (pH 5.5) was then added to the lower buffer chamber and electrophoresis continued at 60W for 2-3 hours.

The gel was removed from the apparatus and washed in a solution of 10% v/v methanol and 10% v/v acetic acid for forty five minutes and dried at 80°C for two hours under vacuum in a flat-bed gel drier. Autoradiography was carried out with an Amersham autoradiography cassette and Amersham Hyperfilm MP with 24 hour exposure at room temperature. The film was kindly developed by the X-ray department at the Royal Infirmary of Edinburgh.

2.11 INDIRECT ANALYSIS OF *GYRA* MUTATIONS.

2.11.1 Restriction of PCR Fragments.

Genome fragments of 17 ciprofloxacin-resistant strains, and PAO1, were amplified by PCR methods (as described in Section 2.9.1). The fragments were ethanol-precipitated and resuspended in 17µl TE buffer. For each restriction digest, 2µl of the appropriate 'REact' buffer (Gibco-BRL) and 1µl of *SstII* restriction enzyme (Gibco-BRL) were added to the PCR fragment, and the mixture incubated at 37°C for three hours. The restricted fragments were then ethanol-precipitated and resuspended in 10µl TE buffer.

2.11.2 Analysis of Restricted DNA.

The restricted fragments were analysed on 1.5% agarose gels by horizontal gel electrophoresis as described in Section 2.8.4 with TAE buffer [10x stock solution per 500ml :- 104g Trizma base; 5.7ml of glacial acetic acid; 10ml of 0.5M Na_2EDTA (pH 8)]. Before analysis of each sample, loading buffer was added to each preparation to create a ratio of loading buffer to sample of 1:6 respectively. The constituents of the loading buffer were :- 0.25% bromophenol blue; 0.25% xylene cyanol; 30% glycerol in sterile distilled water.

Electrophoresis was performed with gels submerged in TAE buffer for 45 minutes at 120 Volts. The gels were stained and visualised as described previously. Restricted fragments were run against controls which contained either a non-restricted PCR fragment, or a PCR fragment incubated in the presence of 'REact' buffer for three hours at 37°C.

2.11.3 Probing for *GyrA* Mutations.

Genetic studies have shown that the quinolone-resistant *gyrA* gene is recessive to the sensitive allele, and a cosmid vector pNJR3-2 (Robillard 1990) has been made to screen bacteria for *gyrA* mediated quinolone resistance. The pNJR3-2 was obtained from Bayer, UK. with the kind permission of N. Robillard, and contains the *E.coli* quinolone-sensitive *gyrA* gene. The pNJR3-2 was used to probe 4 high level quinolone-resistant isolates of *P.aeruginosa*. A high copy number plasmid, pUC19, was used as a negative control to ensure that the act of conjugation itself did not affect quinolone resistance. Minipreparations of plasmid DNA were obtained by the alkaline lysis method of Takahashi and Nagano (1984), from 4.5ml of cells grown overnight in nutrient broth at 37°C with vigorous shaking.

The conjugations were carried out by the method of Robillard (1990). In brief, the *E.coli* donor strain containing pNJR3-2, and the *P.aeruginosa* recipient strains were grown in ML broth (per litre:- 10g Bacto tryptone (Difco), 5g Bacto yeast extract (Difco), 5g NaCl and 2g K_2HPO_4.) overnight, with aeration at 37°C. The donor strain was grown in the presence of 5µg/ml tetracycline. Five hundred microlitres of donor culture was added to 0.5ml recipient culture and centrifuged for half a minute at 13,000rpm in a microcentrifuge. The mating mixture was resuspended in 50µl of 0.15M NaCl and plated onto ML agar [ML broth containing 1.5% Bacteriological Agar No. 1 (Difco)].

Mating was allowed to occur during a five hour incubation period at 37°C, and the mating mixture was harvested in 3ml of 0.15M NaCl. The mixture was then plated onto ML agar containing 400, 500 or 600µg of tetracycline per ml, and plates were incubated for 24 or 48 hours at 37°C. Conjugants were purified on selection media. Control reactions containing pUC19 were plated onto ML agar containing 100µg of ampicillin per ml.

Conjugation experiments were also carried out as described by Amyes and Gould (1984). In these experiments donor and recipient strains were grown statically overnight at 37°C. Then, either 0.1ml of donor culture was mixed with 1ml of recipient culture in 4.5ml nutrient broth, or 1ml of the donor strain was mixed with 1ml of the recipient culture in 4.5ml nutrient broth. The mixtures were then incubated at 37°C for six hours. Mating mixtures containing *E.coli* with pNJR3-2 and *P.aeruginosa* strains were plated onto IST agar containing 400 and 600µg/ml tetracycline; whereas mating mixtures containing *E.coli* with pNJR3-2 and the *E.coli* strain J62-2 were plated onto DM agar plates containing rifampicin at 25µg/ml.

2.11.4 Transformation of *P.aeruginosa* by Electroporation.

Three ciprofloxacin-resistant clinical isolates of *P.aeruginosa*, 4124, 4125 and 3, and the sensitive strain PAO1 were electroporated with either pNJR3-2 or pUC18, which was used as a negative control. Plasmid DNA was prepared by the method of Takahashi and Nagano (1984). Electroporation was performed with an Electrocell Manipulator 6000 (BTX.) according to the method of Diver *et al* (1990a). Strains were grown overnight in 500ml of LB broth and immediately placed on ice. Cells were harvested by centrifugation at 5,500rpm for 15 minutes and the pellet washed three times in 1mM Hepes buffer. The final pellet was resuspended in 7ml of HGEB. All cell washing procedures were carried out on ice.

Aliquots of 50µl of the mixture were placed into 1.5ml microcentrifuge tubes and 5µl of either pUC18 or pNJR3-2 added. The same volume of buffer was added to a control tube. Tubes were placed on ice for one minute and transferred to prechilled cuvettes with a 0.1cm electrode gap. The electrical settings of the Electrocell Manipulator were as follows:- set voltage 1.4kV; discharge capacitor 25µF; and pulse controller parallel resistor 246Ω (giving a theoretical time constant of between 5 and 10 milliseconds).

The capacitor was discharged through the sample, and immediately after discharge 950µl of ice cold SOC broth was added. Samples were incubated on ice for 30 minutes, transferred back to their tubes and incubated at 37°C for 90 minutes. Aliquots of 50µl and100µl of the samples were plated onto IST agar containing 150µg/ml tetracycline and incubated at 37°C for 48 hours. Samples containing the plasmid pUC18 were selected on IST agar plates containing 50µg/ml ampicillin. Transformants were purified by selection on the appropriate media.

RESULTS.

3.1 MINIMUM INHIBITORY CONCENTRATIONS.

Minimum inhibitory concentrations (MICs) measure the amounts of various antimicrobial agents required to inhibit completely the growth of the target organism, and thus allow comparisons between bacterial strains. MICs may be determined by agar dilution or broth dilution techniques. In the case of the 4-quinolones, MICs must be determined by agar dilution as filamentation of bacteria, caused by quinolones, can lead to erroneus MIC values in broth dilution techniques (Smith 1984b).

3.1.1 Determination of MICs of Quinolones Against *P.aeruginosa*.

STRAIN	MIC mg/l	
	Ciprofloxacin	Ofloxacin
PAO1	2	4
U423	0.5	4
C1	64	128
C2	64	>128
C32	32	128
C48	128	>128
C49	32	32
3	>128	>128
48	16	64
273	32	>128
274	16	64
4124	>128	>128
4125	>128	>128
4149	16	64
4161	64	>128
4269	64	>128

Table 3.1. Minimum Inhibitory Concentrations of Quinolones on PIA.

MICs of ciprofloxacin, ofloxacin and norfloxacin were determined by employing a doubling dilution method against 14 ciprofloxacin-resistant and 2 ciprofloxacin-sensitive strains on *Pseudomonas* Isolation Agar (PIA), and against 25 ciprofloxacin-resistant and 4 ciprofloxacin-sensitive strains on Isosensitest Agar (IST) (Tables 3.1 and 3.2).

STRAIN	MIC mg/l		
	Ciprofloxacin	Ofloxacin	Norfloxacin
PAO1	0.5	1	2
U423	0.25	1	-
C1	16	64	>128
C2	32	32	64
C32	8	32	64
C48	32	64	>128
C49	8	8	32
3	64	128	>128
48	2	16	-
52	4	8	32
135	2	8	-
271	8	64	128
273	8	64	64
274	4	8	32
4124	64	128	>128
4125	64	>128	>128
4131	16	64	>128
4133	64	128	>128
4149	8	32	128
4158	16	32	64
4161	16	64	128
4269	16	128	128
4352	16	32	128
4374	16	64	128
4375	8	64	128
4495	16	64	128
4532	8	64	>128
5762	0.25	-	-
6397	0.25	-	-

- not done

Table 3.2. Minimum Inhibitory Concentrations of Quinolones on IST Agar.

Strains were classified as quinolone-resistant if they exhibited an MIC to quinolones of 4mg/l or greater, and quinolone-sensitive if they demonstrated an MIC of less than 2mg/l. In all strains, resistance to ciprofloxacin resulted in resistance to all other quinolones tested. Ciprofloxacin was the most active quinolone, being at least twice as active as ofloxacin and four to eight times as active as norfloxacin in terms of MIC (Tables 3.3 and 3.4).

STRAIN	MIC mg/l			RATIO OF DRUG RESISTANCE TO CIPROFLOXACIN RESISTANCE	
	Ciprofloxacin	Ofloxacin	Norfloxacin	Ofloxacin	Norfloxacin
U423	0.25	1	-	4	-
PAO1	0.5	1	2	2	4
48	2	16	-	8	-
135	2	8	-	4	-
52	4	8	32	2	8
274	4	8	32	2	8
C32	8	32	64	4	8
C49	8	8	32	1	4
271	8	64	128	8	16
273	8	64	64	8	8
4149	8	32	128	4	16
4375	8	64	128	8	16
4532	8	64	>128	8	>16
C1	16	64	>128	4	>8
4131	16	64	>128	4	>8
4158	16	32	64	2	4
4161	16	64	128	4	8
4269	16	128	128	8	8
4352	16	32	128	2	8
4374	16	64	128	4	8
4495	16	64	128	4	8
C2	32	32	64	1	2
C48	32	64	>128	2	>4
3	64	128	>128	2	>2
4124	64	128	>128	2	>2
4125	64	>128	>128	>2	>2
4133	64	128	>128	2	>2

Table 3.3. Ratios of Quinolone MICs on IST Agar.

STRAIN	MIC mg/l		DRUG RATIO Cipro./ Oflox.
	Ciprofloxacin	Ofloxacin	
U423	0.5	4	8
PAO1	2	4	2
48	16	64	4
274	16	64	4
4149	16	64	4
C32	32	128	4
C49	32	32	1
273	32	>128	>4
C1	64	128	2
C2	64	>128	>2
4161	64	>128	>2
4269	64	>128	>2
C48	128	>128	>1
3	>128	>128	-

Table 3.4. Relationship of Ciprofloxacin MIC to Ofloxacin MIC on PIA.

The ciprofloxacin/ofloxacin and the ciprofloxacin/norfloxacin ratios, however, were not constant within the groups studied, which indicates that several different quinolone resistance mechanisms might be occurring within these strains. There was no significant difference seen in the ciprofloxacin ratios for strains investigated on both PIA and IST agar. Resistant strain 4158 exhibited the same ciprofloxacin/ofloxacin and ciprofloxacin/norfloxacin ratios as the sensitive strain PAO1 (Table 3.3), whereas most other strains had considerably higher ratios (Tables 3.3 and 3.4).

Strains C49 and C2 exhibited lower ciprofloxacin/ofloxacin and ciprofloxacin/norfloxacin ratios than the sensitive strains (Tables 3.3 and 3.4); and both showed no alteration in MIC between ciprofloxacin and ofloxacin, which all other

strains exhibited, although when MICs were carried out on PIA, C2 exhibited an increase in the ciprofloxacin/ofloxacin drug ratio which was not reflected in the IST agar results. This may be caused by the detergent present in the PIA interfering with the results. Several strains demonstrated similar ratios despite having different initial MIC values; 52, 274 and 4352 being in one group (Table 3.3), and C32, 4374 and 4495 being in another (Table 3.3). This suggests that these strains may be exhibiting the same quinolone resistance mechanisms.

All quinolone MICs were less active in terms of MIC on IST agar compared with PIA (Tables 3.3 and 3.4). This may have been caused by the detergent in the PIA interfering with the quinolone antibacterials. Many of the clinical isolates exhibited mucoidy on PIA and not IST agar, termed PIA dependent mucoidy (Govan *et al* 1983), and this may have contributed to the greater MIC values on this media.

3.1.2 Determination of MICs of Other Antimicrobial Agents Against *P.aeruginosa*.

Quinolone resistance in *P.aeruginosa* has been linked to cross resistance with imipenem, carbenicillin, novobiocin and chloramphenicol and hypersusceptibility to gentamicin and β-lactam antibiotics. This documented cross resistance to other antimicrobial agents is brought about by permeability mutations, generally altering the pore size of porin molecules, or by causing decreased expression of certain porin molecules upon the cell surface. As the mechanism of entry of many antimicrobial agents into the bacterial cell is passive, involving diffusion of the agent through porin channels, any alteration in porin channels is likely to bring about resistance to several antimicrobial agents in one step. Therefore MICs of imipenem, cefotaxime, ceftazidime, gentamicin, carbenicillin, tetracycline and kanamycin were determined by a doubling dilution method for 27 strains on IST agar (Table 3.5).

STRAIN	MIC mg/l						
	IM	CTX	CAZ	GN	CAB	TET	KM
PAO1	2	8	2	2	64	64	8
U423	0.5	16	2	2	64	-	-
C1	0.5	64	8	4	>128	128	32
C2	4	64	4	8	>128	64	128
C32	0.5	64	8	4	>128	256	8
C48	0.25	8	1	0.5	4	>256	8
C49	1	16	2	2	32	128	32
3	1	64	4	2	128	128	64
48	2	8	2	0.5	32	-	8
52	1	32	4	1	128	128	2
135	8	4	4	1	32	-	-
271	1	16	2	4	32	64	16
273	4	64	8	2	>128	128	64
274	2	16	4	4	64	64	32
4124	1	32	2	4	64	128	32
4125	1	32	2	4	64	128	32
4131	1	32	8	16	>128	128	128
4133	4	32	4	64	>128	128	>128
4149	4	64	8	128	>128	128	64
4158	0.5	16	2	2	32	128	64
4161	0.5	8	1	2	32	128	16
4269	0.5	128	4	2	>128	128	64
4352	4	4	2	4	4	256	128
4374	4	16	2	32	16	256	>128
4375	8	64	8	128	>128	128	64
4495	4	128	8	2	32	64	32
4532	2	8	1	1	16	128	16

IM- imipenem, CTX- cefotaxime, CAX- ceftazidime, GN- gentamicin, CAB- carbenicillin, TET- tetracycline, KM - kanamycin.

Table 3.5. Minimum Inhibitory Concentrations of *P.aeruginosa* to Other Antimicrobial Agents.

According to the break point standards recommended by the Working Party of the British Society for Antimicrobial Chemotherapy (1991), all strains were found to be tetracycline-and kanamycin-resistant, with the exception of strain 52 which exhibited kanamycin sensitivity. The sensitive strains PAO1 and U423 exhibited high resistances to cefotaxime, carbenicillin, tetracycline, and in PAO1, kanamycin, indicating that all strains of *P.aeruginosa* are likely to be intrinsically resistant to these antimicrobial agents. Most strains exhibited a higher resistance to cefotaxime than the sensitive

strains, although two strains, 4352 and 135, had only a moderate resistance of 4mg/l. Strains C48, 48, 4161 and 4532 exhibited identical levels of cefotaxime resistance to the sensitive strains. Similar results were observed for ceftazidime, gentamicin, carbenicillin and imipenem.

Most strains were also resistant to carbenicillin with MICs of greater than 32mg/l, although strains 4352 and C48 exhibited MIC values of 4mg/l and strains 4374 and 4532 were observed to have MICs of 16mg/l. Seven other strains were observed to have carbenicillin MICs lower than the sensitive strains PAO1 and U423.

MICs of imipenem, ceftazidime and gentamicin were variable, with 29% strains exhibiting imipenem resistance of greater than 2mg/l; 46% had ceftazidime resistance of greater than 2mg/l and 46% had gentamicin resistance of greater than 2mg/l. Only four strains, 4375, 4149, 4133 and C2, however, showed resistance to all three drugs, with most of the other resistant strains exhibiting resistance to combinations of two of the antimicrobial agents. Strains 3, 52 and 4269 exhibited moderate resistance to ceftazidime only, and strains 271, 4124 and 4125 exhibited moderate resistance to gentamicin only.

In all cases, resistance to ceftazidime was linked to high levels of resistance to cefotaxime, although strains with the same ceftazidime MIC had varying cefotaxime MICs. Strains 4124 and 4125 had a ceftazidime MIC of 2mg/l, on the break point for resistance, but exhibited high level resistance to cefotaxime. It might be the case that these strains were slightly ceftazidime-resistant, but that the experimental technique was not suitable to reflect this.

Of the 13 gentamicin-resistant strains, three strains, 4375, 4149 and 4133, were highly resistant to the drug with MICs greater than 64mg/l. These three strains also exhibited high MIC levels to all antimicrobial agents tested, suggesting that resistance was probably caused by a major membrane protein alteration. Strains 4375 and 4149 showed moderate quinolone resistance, whereas 4133 exhibited high level quinolone resistance.

Six strains, C1, C32, C48, 4158, 4161 and 4269 exhibited imipenem sensitivity comparable to the sensitive strain U423. Strains 4269 and C1 exhibited no sensitivity to any other antimicrobial agent; strains 4158 and 4161 both exhibited lower carbenicillin MICs than the sensitive strains and, in addition, 4161 was also ceftazidime-sensitive. Strains C32 and C48 were observed to have identical kanamycin sensitivities to PAO1, and C48 was also sensitive to all other antimicrobial agents tested, with the exception of tetracycline and cefotaxime.

To summarise, the ciprofloxacin-resistant strains exhibited varying antimicrobial sensitivity patterns, suggesting that the strains possessed differing quinolone resistance mechanisms. These results also suggest that the strains with resistance to ceftazidime, gentamicin and imipenem exhibit varying resistance mechanisms, as not all strains possess resistance to all three antimicrobial agents. Resistance to carbenicillin, tetracycline, cefotaxime and kanamycin is unlikely to be related to quinolone resistance, as the quinolone-sensitive strains were also resistant to these drugs.

3.1.3 Antimicrobial Sensitivity of Paired Clinical Isolates of *P.aeruginosa*.

MICs of ciprofloxacin, ofloxacin, norfloxacin, gentamicin, imipenem, cefotaxime, ceftazidime and carbenicillin were determined for a series of paired clinical isolates (Table 3.6). These isolates had developed ciprofloxacin resistance during therapy at Glasgow Royal Infirmary, and were from three separate patients (refer to Materials and Methods Section 2.2). The relationship of resistance between the various 4-quinolones tested was also investigated (Table 3.7).

STRAIN	MIC mg/l						
	CIP	OFX	GN	CAZ	IM	CTX	CAB
91-32	0.25	1	1	2	2	8	32
91-33	1	4	2	2	2	8	32
91-41	1	4	1	8	2	128	64
91-40	1	8	1	8	2	128	64
91-42	1	8	2	32	4	>128	64
91-44	1	8	2	64	4	>128	128
91-46	2	8	4	64	4	>128	128
91-76	2	8	2	8	4	128	64
91-28	0.5	4	2	2	4	16	64
91-36	2	2	2	2	4	16	64
90-62	1	4	2	2	2	16	32
90-67	4	8	2	32	2	>128	64

CIP- ciprofloxacin, OFX- ofloxacin, GN- gentamicin, CAZ- ceftazidime, IM- imipenem, CTX- cefotaxime, CAB- carbenicillin.

Table 3.6. Minimum Inhibitory Concentrations in Paired Clinical Isolates of *P.aeruginosa*.

As seen with the quinolone-resistant strains previously, ciprofloxacin resistance in the three groups of paired isolates resulted in resistance to the other 4-quinolones, norfloxacin and ofloxacin (Tables 3.6 and 3.7). Ciprofloxacin was observed to be the most active 4-quinolone as it was four times more active than ofloxacin or norfloxacin. Strains 91-36 and 90-67, however, exhibited a two-fold decrease in ofloxacin activity

compared to ciprofloxacin. In all cases, resistance to ofloxacin and ciprofloxacin was similar (Table 3.7), which conflicts with the results obtained when investigating the unpaired clinical isolates previously (Table 3.3), and suggests that a single mechanism was probably responsible for the development of low level quinolone resistance in the paired isolates.

STRAIN	MIC mg/l			Ratio of drug resistance to ciprofloxacin resistance	
	Ciprofloxacin	Ofloxacin	Norfloxacin	Ofloxacin	Norfloxacin
91-32	0.25	1	1	4	4
91-33	1	4	4	4	4
91-41	1	4	8	4	8
91-40	1	8	4	8	4
91-42	1	8	8	8	8
91-44	1	8	4	8	4
91-46	2	8	8	4	4
91-76	2	8	8	4	4
91-28	0.5	4	4	8	8
91-36	2	8	16	4	8
90-62	1	4	4	4	4
90-67	4	8	16	2	4

Table 3.7. Ratios of Quinolones to Ciprofloxacin on PIA, in Paired Clinical Isolates.

As observed with the unpaired quinolone-resistant isolates, all of the paired strains exhibited cefotaxime and carbenicillin resistance, again suggesting that *P.aeruginosa* is intrinsically resistant to these antimicrobial agents (Table 3.6). All of the paired isolates exhibited stable gentamicin and imipenem resistance, which was also comparable between groups. In the case of the paired strains 90-62 and 90-67, acquisition of resistance to quinolones was accompanied by resistance to the β-lactam drug ceftazidime, although whether this resistance developed independently of quinolone resistance was unclear. Resistance to ceftazidime was also accompanied by an increased resistance to another β–lactam antibiotic, cefotaxime.

In the series 91-32 to 91-76, from Patient I, initial quinolone resistance was not accompanied by cross resistance to any other antimicrobial agent. Later isolates, from 91-41 to 91-76, subsequently developed resistance to ceftazidime, which increased throughout the series from four- to 32-fold. Again, increases in ceftazidime resistance brought about increased resistance to cefotaxime. The strain 91-76 exhibited lower ceftazidime resistance than previous isolates.

The paired isolates 91-28 and 91-36 demonstrated an increase in quinolone resistance which was not linked to cross resistance to any other antimicrobial agents. These results confirm previous suggestions that at least two quinolone resistance mechanisms are present in the strains investigated; those that produce resistance solely to 4-quinolones, and those which cause quinolone resistance accompanied by cross resistance to other antimicrobial agents, particularly β-lactam antibiotics.

3.2 STABILITY OF QUINOLONE RESISTANCE.

It has been reported that quinolone resistance in *E.coli* and *P.aeruginosa* is reduced after repeated passage on drug-free media (Daikos *et al* 1988). Conflicting reports (Rådberg *et al* 1990) have established little or no alteration in ciprofloxacin resistance in *P.aeruginosa* after serial passage in Mueller-Hinton broth. To investigate ciprofloxacin stability in clinical isolates, 20 ciprofloxacin-resistant strains were passaged daily onto drug-free PIA, with a single colony isolated for each passage. Ciprofloxacin MICs of the 20 strains were investigated before the initial passage, and subsequently after every five passages. After 15 successive passages of the strains on drug-free PIA, there was little or no decrease in the MIC of ciprofloxacin on IST agar (Table 3.8).

STRAIN	MIC of ciprofloxacin mg/l after passage number:- 0	5	10	15
PAO1	0.5	0.5	0.5	0.5
U423	0.25	0.25	0.25	0.25
C1	16	4	4	8
C2	32	8	8	8
C32	8	8	4	8
C48	32	0.25	0.25	0.25
C49	8	8	4	4
3	64	64	64	128
48	2	4	4	4
271	8	4	4	8
273	8	8	16	16
274	4	4	4	4
4124	64	64	64	64
4125	64	64	64	64
4131	16	16	32	32
4133	64	64	32	64
4269	16	16	16	16
4352	16	8	8	8
4374	16	8	8	8
4495	16	8	8	16

Table 3.8. Stability of Clinical Isolates to Ciprofloxacin After Passage on Drug-Free PIA.

After 5 passages, some strains exhibited a slight decrease in ciprofloxacin MIC. Strains C1 and C2 exhibited a four-fold decrease in ciprofloxacin MIC, which after 15 passages in strain C1 resulted in a final two-fold decrease in overall resistance. Strain C2 maintained a four-fold decrease in ciprofloxacin resistance throughout the experiment. Strains 271, 4352, 4374 and 4495 demonstrated a two-fold decrease in ciprofloxacin resistance, which was recovered in strains 4495 and 271 after 15 passages. In one strain, 48, an increase in ciprofloxacin resistance was observed after 5 passages, which remained stable throughout the remainder of the experiment and suggested that the initial ciprofloxacin MIC might have been too low.

After 10 passages on drug-free media, C32, C49 and 4133 exhibited a two-fold decrease in ciprofloxacin MIC, whilst 4131 exhibited a two-fold increase in MIC, which was maintained until the end of the experiment. The significance of the ciprofloxacin MIC alterations observed must be questioned, as the MICs of the relevant strains are still classified as resistant by the break-point criteria of the Working Party of the British Society for Antimicrobial Chemotherapy (1991), and in most cases the initial ciprofloxacin resistance was retained.

Resistance was found to be stable in all strains, with one exception. In strain C48, resistance after 5 passages decreased over one hundred-fold, indicating that ciprofloxacin resistance in this strain was unstable in the absence of the drug. The subsequent strain, C48s, was shown to have the same pyocin type as its parent strain, and remained sensitive throughout the experiment. On repeating the same experiment with C48, no reversion to sensitivity was observed. This result suggests that the same strain may exhibit two different mutations, one of which is stable in the absence of ciprofloxacin, and one which is unstable in the absence of the selective agent.

3.3 ALTERATIONS IN OUTER MEMBRANE PROTEINS.

Previous investigations have shown that quinolone resistance in *P.aeruginosa* may be associated with alterations in outer membrane proteins (OMPs) (Rella and Haas 1982; Hirai *et al* 1987; Legakis *et al* 1989). These changes involve alterations in proteins of 32kD, 50kD, 51kD and 54kD, and may also involve cross resistance to imipenem and other antimicrobial agents (Rådberg *et al* 1990; Fukuda *et al* 1990). From the MIC results of the quinolone-resistant unpaired clinical isolates, it seems likely that most strains would show OMP alterations. Strains exhibiting imipenem resistance, C2, 135, 273, 4133, 4149, 4374, 4375 and 4495 would be expected to carry OMP alterations, as would those strains with high β-lactam resistance; C1, C32, 3, 52, 4131 and 4269. Other strains demonstrating gentamicin resistance or carbenicillin hypersusceptibility might also harbour OMP alterations. As β-lactam resistance and imipenem resistance are not linked in the majority of cases, strains exhibiting resistance to both antimicrobial agents might be expected to harbour more than one OMP alteration.

The results from the paired strains indicate that 91-33, and subsequent strains in that series, 91-36 and 90-67 should have altered OMP profiles compared to their parent strains, as low level quinolone resistance has been linked with OMP alterations. Strain 91-41 and subsequent strains might also be expected to display another OMP alteration from the parent strain, 91-32, and the quinolone-resistant strain 91-33, as they have developed β-lactam resistance. Strain 90-67 might also be expected to display a similar OMP alteration to 91-41, as it has also developed β-lactam resistance.

In order to investigate these assumptions, the OMPs of both paired and unpaired isolates of *P.aeruginosa* were examined. OMPs were prepared, analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by Coomassie brilliant blue R staining. The OMPs were run, initially, on the PhastSystem® with a 10-15% polyacrylamide gel, but band differentiation was poor in

the lower protein molecular mass areas, as the gels were so small. The samples were also run on the BioRad Mini Protean® system on larger gels, with a fixed polyacrylamide gel gradient, and as this system showed improved band differentiation it was retained for the remainder of the samples. Proteins were run against molecular mass standards, the sizes of which are given in Table 3.9.

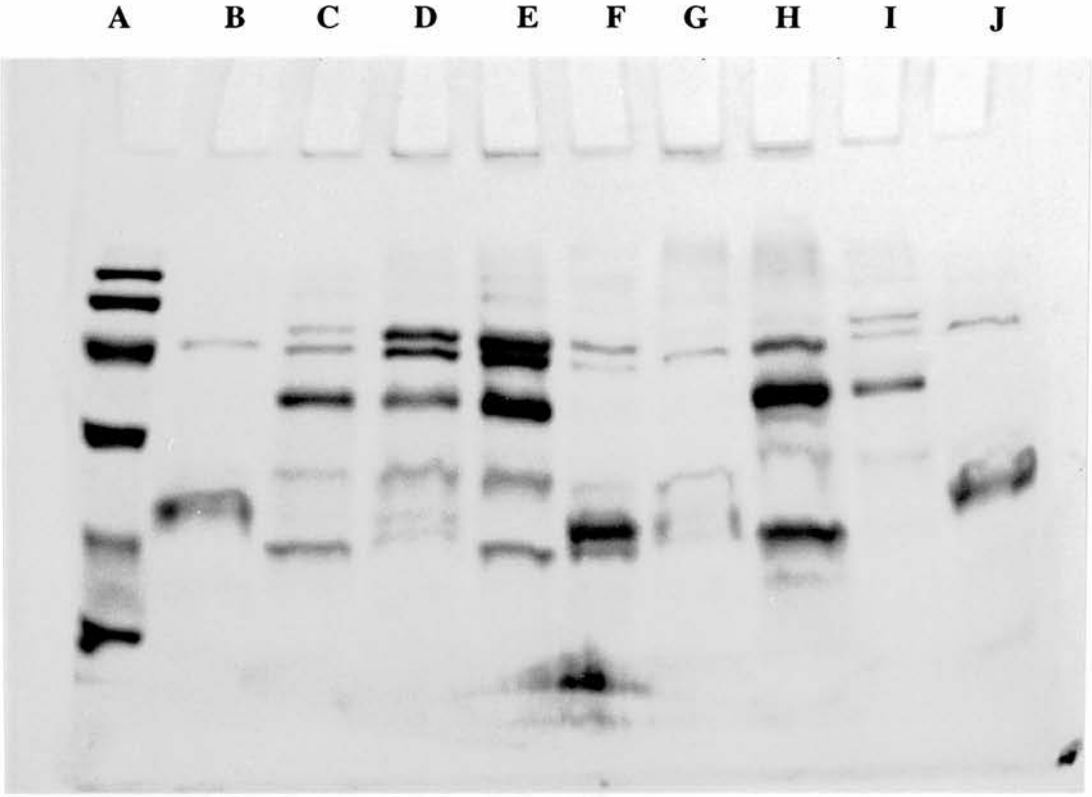
Molecular Mass Standards	(Daltons)
Phosphorylase B	94,000
Bovine Serum Albumin	67,000
Ovalbumin	43,000
Carbonic Anhydrase	30,000
Soybean Trypsin Inhibitor	20,100
α-Lactalbumin	14,400

Table 3.9. Molecular Masses of Standards Run on SDS-PAGE.

The OMPs of quinolone sensitive isolates were investigated to provide a comparison for the resistant strains. The OMPs of 8 quinolone-sensitive isolates of *P.aeruginosa* were separated by SDS-PAGE, using the Mini Protean® system, and visualised by Coomassie brilliant blue R staining. The results are shown in Figure 3.1. The sensitive strains were observed to have varied OMP profiles, although all strains possessed a protein at 46kD. Strains C48s (lane B), PAO1 (lane G) and 135 (lane J) exhibited similar profiles with two proteins at 46kD and 26kD.

Fig 3.1. OMP Profile of Sensitive Strains of *Pseudomonas aeruginosa.*

Tracks are :- Lane A, molecular mass standards: Lane B, C48s: Lane C, 5762: Lane D, 6397: Lane E, U423: Lane F, 91-32: Lane G, the reference strain PAO1: Lane H, 91-28: Lane I, 8252: Lane J, 135.



Five strains exhibited another basic profile, with varying amounts of the proteins expressed, although this is likely to be a reflection of the varying concentrations of the preparations used. Strains 5762, 6397, U423 (lanes C-E), 91-28 (lane H) and 8252 (lane I) were observed to have similar profiles, with 91-28 possessing a very weak 51kD protein and 8252 lacking a protein at 22kD. Strain 91-32 (lane F) displayed a profile which lacked a 36kD protein and had an extra protein at 24kD. As the OMP profile of U423 was displayed by a majority of sensitive strains it was used as the main comparison for the resistant isolates.

To improve the comparison of OMPs with resistant isolates, mutant bacteria were selected from strain U423. U423 was seeded onto PIA containing ciprofloxacin at 5 times the MIC and incubated overnight. The mutational frequency was observed to be 6.1 in 10^5. Six mutant strains were selected from the ciprofloxacin containing plate, and the MICs of a range of antibiotics for each of the mutant strains were determined and compared to those of the parent strain in Table 3.10. All the mutant isolates exhibited an eight-fold increase in ciprofloxacin resistance, compared to the parent strain, and were also pigmented; strains U423A and C being yellow and strains U423B, D, E and F being green.

Pyocin typing indicated that the mutant strains were related to the parent strain (refer to Appendix i). As the mutant strains produced pigments not present in the parent strain, it was likely that the pigment production interfered with the pyocin-typing procedure, so the mutants did not exhibit identical pyocin profiles to the parent strain. In all cases pigment production remained stable in the absence of the selective pressure.

STRAIN	MIC mg/l						
	CIP	OFX	GN	CAZ	IM	CTX	CAB
U423	0.25	1	1	2	2	8	32
U423A	2	8	1	1	2	8	32
U423B	2	8	0.25	1	0.25	8	8
U423C	2	8	1	1	2	8	32
U423D	2	8	1	2	0.5	8	32
U423E	2	8	0.5	2	0.5	8	32
U423F	2	8	2	2	0.25	8	16

CIP- ciprofloxacin, OFX- ofloxacin, GN- gentamicin, CAZ- ceftazidime, IM- imipenem, CTX- cefotaxime, CAB- carbenicillin.

Table 3.10. Minimum Inhibitory Concentrations of Laboratory Mutants of U423.

The mutant strains exhibited similar β-lactam resistance to the parent strain U423. The two yellow-pigmented strains, U423A and U423C, exhibited identical antibiograms and also possessed identical pyocin types. The green-pigmented strains all exhibited imipenem sensitivity, which has been previously linked to quinolone resistance (Rådberg *et al* 1990). The mutants U423B and E also demonstrated gentamicin sensitivity, and U423B displayed a lower carbenicillin MIC than the quinolone-sensitive parent strain, U423.

As the mutants U423A and U423C exhibit antibiograms that are virtually identical to the parent strain U423, except for the quinolone MICs, they might be expected to exhibit quinolone resistance mechanisms other than permeability alterations, and so would demonstrate identical OMP profiles to U423. The other mutant strains U423B, D, E and F have acquired quinolone resistance linked to imipenem sensitivity, and other drug sensitivities, and may exhibit OMP alterations.

In order to investigate this further, the OMP profiles of the mutant strains were compared to that of U423 by SDS-PAGE (Fig 3.2). All isolates exhibited identical OMP profiles, but the resistant isolates appeared to have a slight increase in a protein of approximately 36kD and two strains, U423E (lane C) and U423D (lane E) exhibited a decrease in a protein of approximately 51kD.

The OMPs of eight clinical ciprofloxacin-resistant isolates were investigated by SDS-PAGE on the Mini Protean® system. Strains were selected as they exhibited high ciprofloxacin resistance and also had varying antibiograms (refer to Tables 3.3 and 3.5). Strains 4124 and 4125 demonstrated ciprofloxacin resistance of 64mg/l and slightly raised cefotaxime resistance of 32mg/l. Strains 4158 and 4161 had ciprofloxacin MIC values of 16mg/l, coupled with imipenem hypersusceptibility, whilst strain 4269 exhibited a similar antibiogram with increased β-lactam resistance. Strains 4352 and 4374 had a ciprofloxacin MIC of 16mg/l with raised imipenem MICs of 4mg/l, and strain 4374 also exhibited a raised gentamicin MIC. Strain 4131 was selected as it exhibited ciprofloxacin resistance of 16mg/l coupled with ceftazidime and gentamicin resistance.

The selected strains demonstrated slight variations in OMP profiles, with a band at 36kD being strongly expressed by them all (Fig 3.3). Strains 4161, 4158, 4124 and 4125 were observed to have a decrease in a 51kD protein, whilst 4352 (lane E) showed a significant increase in the production of an identical protein. In strains 4374, 4352, 4125 and 4124 a double band at 38kD and 36kD was observed. Strains 4124 and 4125 also exhibited extra protein bands at 32kD and 28kD. A band at 17kD was strongly expressed in 4161, 4158 and 4133, and was also present in most of the remaining strains, with the exception of 4374 and 4352.

Figure 3.2. OMP Profile of U423 and its Mutant Strains.

Tracks include :- Lane A, the parent strain U423: Lane B, U423F: Lane C, U423E: Lane D, U423: Lane E, U423D: Lane F, U423C: Lane G, U423: Lane H, U423B: Lane I, U423A: Lane J, Molecular mass standards.

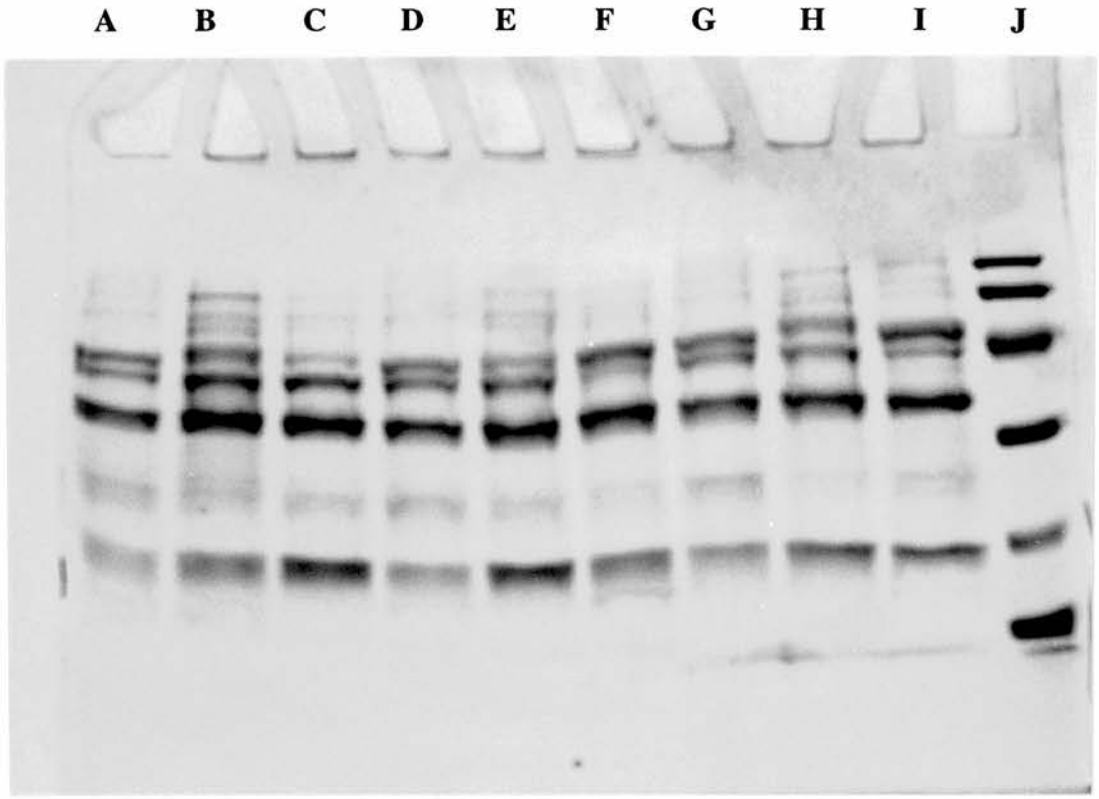
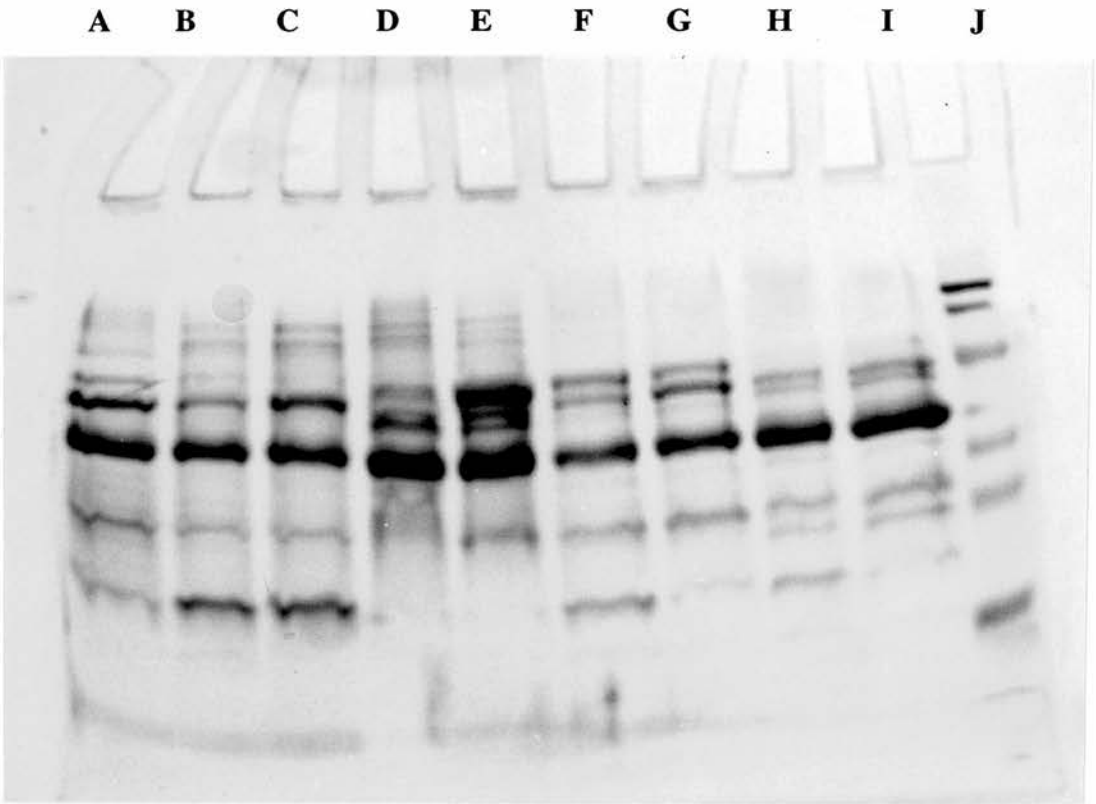


Figure 3.3. OMP Profiles of Highly Resistant Clinical Isolates.

Tracks include :- Lane A, 4269: Lane B, 4161: Lane C, 4158: Lane D, 4374: Lane E, 4352: Lane F, 4133: Lane G, 4131: Lane H, 4125: Lane I, 4124: Lane J, Molecular mass standards.



The strongly-expressed 36kD protein is also expressed in sensitive strains, but to a lesser extent. It is also the identical protein to the protein which was found to express itself to a greater extent in the ciprofloxacin-resistant laboratory mutants of U423. These results suggest that the 36kD protein might be linked to quinolone resistance.

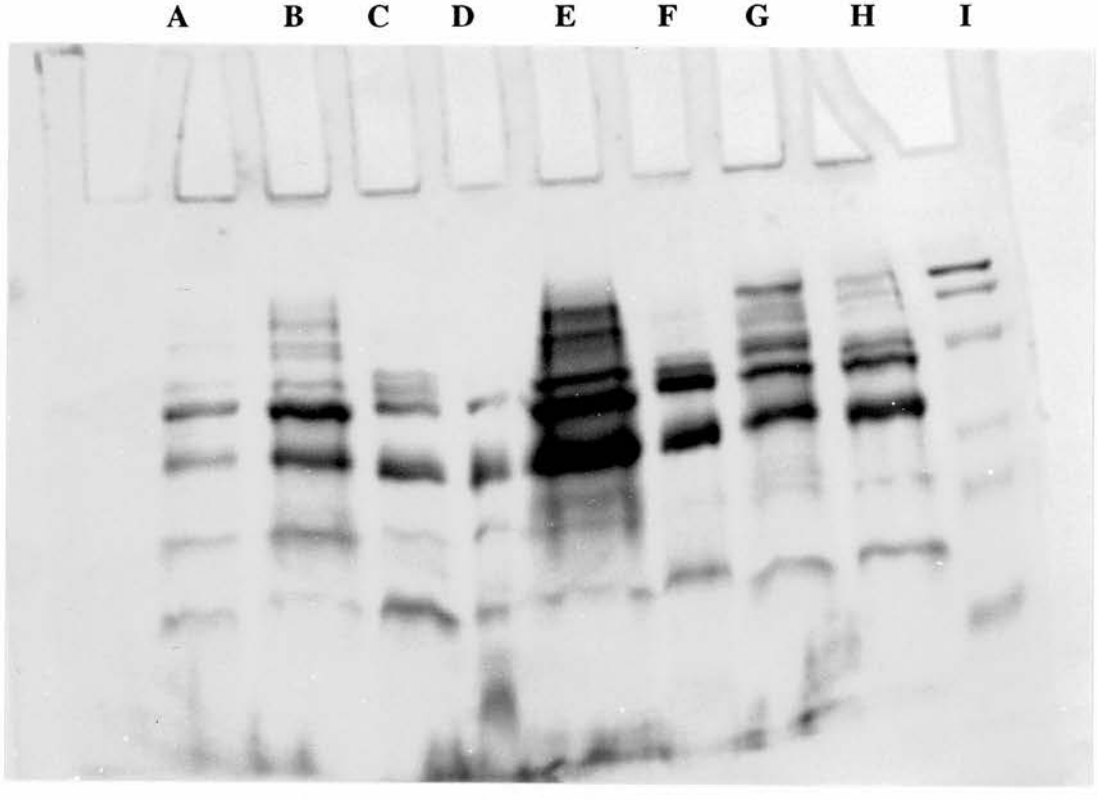
Growing bacteria in the presence of ciprofloxacin has been observed to highlight OMP alterations, when compared to the identical strain grown in the absence of the drug (C.S. Lewin personal communication). Four ciprofloxacin-resistant strains were grown in nutrient broth in the presence of 1/4 MIC ciprofloxacin and their OMP profiles compared to those of strains grown in drug-free medium by SDS-PAGE (Fig 3.4). In all cases no visible differences in OMP profiles between the relevant pairs were observed, which suggests that growth in ciprofloxacin-containing media may only enhance OMP alterations in some strains.

Analysis of the OMPs of all of the ciprofloxacin-resistant strains demonstrated several alterations compared to the sensitive strains screened, and these are listed in Table 3.11. Many of the resistant isolates exhibited alterations in similar OMPs, with decreases in the expression of, or total loss of, a 51kD protein predominating. This alteration was linked, in many cases, to alterations in other OMPs; a common link being a decrease in the expression of the 51kD protein coupled with the production of a new 28kD protein.

Several strains produced an extra 38kD protein in conjunction with alterations in the 51kD protein, although strains C48, 4269 and 4352 produced the new protein without visible alterations in any other OMPs. Another frequent OMP alteration seen, when compared to the sensitive isolates, was the loss of a 43kD protein. Proteins which were altered in a minority of strains include those at 46kD, 32kD and 30kD.

Figure 3.4. OMPs of Strains Grown in the Presence and Absence of Ciprofloxacin.

Tracks include:- Lane A, strain 3 grown in the presence of ciprofloxacin: Lane B, strain 3 grown in the absence of ciprofloxacin: Lane C, strain C1 grown with ciprofloxacin: Lane D, strain C1: Lane E, C32 grown with ciprofloxacin: Lane F, C32: Lane G, C2 grown with ciprofloxacin: Lane H, C2: Lane I, molecular mass standards.



STRAIN	ALTERATION IN OMPS COMPARED TO SENSITIVE STRAINS
C48	Extra 38kD protein.
3	Loss 51kD protein, extra 28kD protein.
52	Decreased expression of 43kD protein.
273	Decreased expression of 51kD protein, increased expression of 43kD protein, loss of 36kD protein.
274	Loss of 46kD and 32kD proteins, extra 42kD and 38kD proteins.
4124	Loss of 51kD protein, extra 38kD, 32kD and 28kD proteins.
4125	Loss of 51kD protein, extra 38kD, 32kD and 28kD proteins.
4131	Loss of 43kD protein, extra 30kD protein.
4133	Loss of 43kD protein, extra 30kD protein.
4158	Loss of 51kD protein.
4161	Decreased expression of 51kD protein.
4269	Extra 30kD protein.
4352	Extra 38kD protein.
4374	Decreased expression of 51kD protein, extra 38kD protein.
4495	Loss of 51kD protein, extra 32kD protein.
4532	Decreased expression of 43kD protein, extra 38kD and 28kD proteins.

Table 3.11. Outer Membrane Protein Alterations Exhibited by Quinolone Resistant Clinical Isolates Compared to Sensitive Isolates.

Strains 4124 and 4125, and 4131 and 4133, showed identical OMP changes, and the pairs 4124 and 4125, and 4131 and 4133, also demonstrated identical pyocin types. These strains were isolated from patients in the same hospitals and are therefore likely to be related.

The major entry route for quinolones into the bacterial cell is through porin pathways (Hirai *et al* 1986b). To investigate whether the alterations in OMPs seen were alterations in porin proteins, several resistant isolates were examined to determine which proteins were non-covalently associated with peptidoglycan, and thus porins. The proteins of 43kD, 46kD and 51kD appeared to be non-covalently associated with peptidoglycan, as they remained with the peptidoglycan fraction after solubilisation with 2% SDS at 37°C (Fig 3.5). These proteins were also released from peptidoglycan by heating in 2% SDS at 100°C for 5 minutes, which suggests that they may function as porins. As the strains investigated were unpaired, however, the changes observed may not be associated with quinolone resistance, but instead may be caused by species variation. To investigate the role of alterations in the 36kD protein further, several paired isolates were examined.

3.3.1 Alterations in OMPs of Paired Isolates.

The OMPs of three sets of paired clinical isolates, a series of eight paired isolates which had developed ciprofloxacin resistance during therapy, and a set of isolates comprised of a clinical strain which reverted to ciprofloxacin sensitivity during stability testing experiments, were examined by SDS-PAGE (Fig 3.6). The OMPs were also investigated for non-covalent association with peptidoglycan to establish whether or not they were porin proteins. The paired isolates 91-28 and 91-36 (lanes B and C, Fig 3.6) exhibited identical OMP profiles, as did strains 8252 and 3221 (lanes F and G), which suggests that quinolone resistance was brought about by other resistance mechanisms.

Ciprofloxacin and β-lactam resistance in the paired isolates 90-62 and 90-67 appeared to be linked to a decrease in the expression of a 46kD protein and increased expression of 26kD and 22kD proteins. The isolates C48, and C48 grown in the presence of ciprofloxacin (lanes H and I), had a completely different profile to the C48 sensitive revertant C48s (lane J), which lacked both a 51kD and a 36kD band. C48s was observed to exhibit decreased expression of a 46kD band and increased expression of a 26kD band.

The OMP profile of C48s bore a strong resemblance to that of another quinolone-sensitive strain, PAO1 (refer to Fig 3.1). The alterations exhibited in these paired isolates mimicked the alterations reported previously in the unpaired resistant clinical strains. As before, the 46kD, 51kD and 36kD proteins appeared to be non-covalently associated with peptidoglycan.

On examining the OMPs of the 91-32 series of paired isolates, ciprofloxacin resistance could be linked to an increase in a 46kD protein and an apparent decrease in a 51kD protein (Fig 3.7), when the ciprofloxacin-resistant strain 91-33 (lane B) was compared to its sensitive parent strain 91-32 (lane A). Ceftazidime resistance in a later isolate, 91-42 (lane E), could also be linked to the appearance of a 42kD protein. Isolates 91-44 and 91-76 appeared to express an extra 36kD protein, which could not be linked to resistance to any of the antimicrobial agents studied.

On further examination, the proteins of 36kD, 42kD, 46kD and 51kD appeared to be associated with peptidoglycan, as they remained with the peptidoglycan fraction after solubilisation with SDS. The association was then shown to be non-covalent, as the proteins were released from the insoluble peptidoglycan fraction by boiling for five minutes. These results suggest that the proteins are probably porins (Fig 3.8).

Figure 3.5. OMP Profiles of Porin Preparations of *P.aeruginosa* Isolates.

Tracks are as follows:- Lane A, strain 274: Lane B, strain 273: Lane C, a sensitive strain U423: Lane D, strain 4495: Lane E, strain 3: Lane F, the reference strain PAO1: Lane G, a sensitive strain 5762: Lane H, a sensitive strain 6397: Lane I, molecular mass standards.

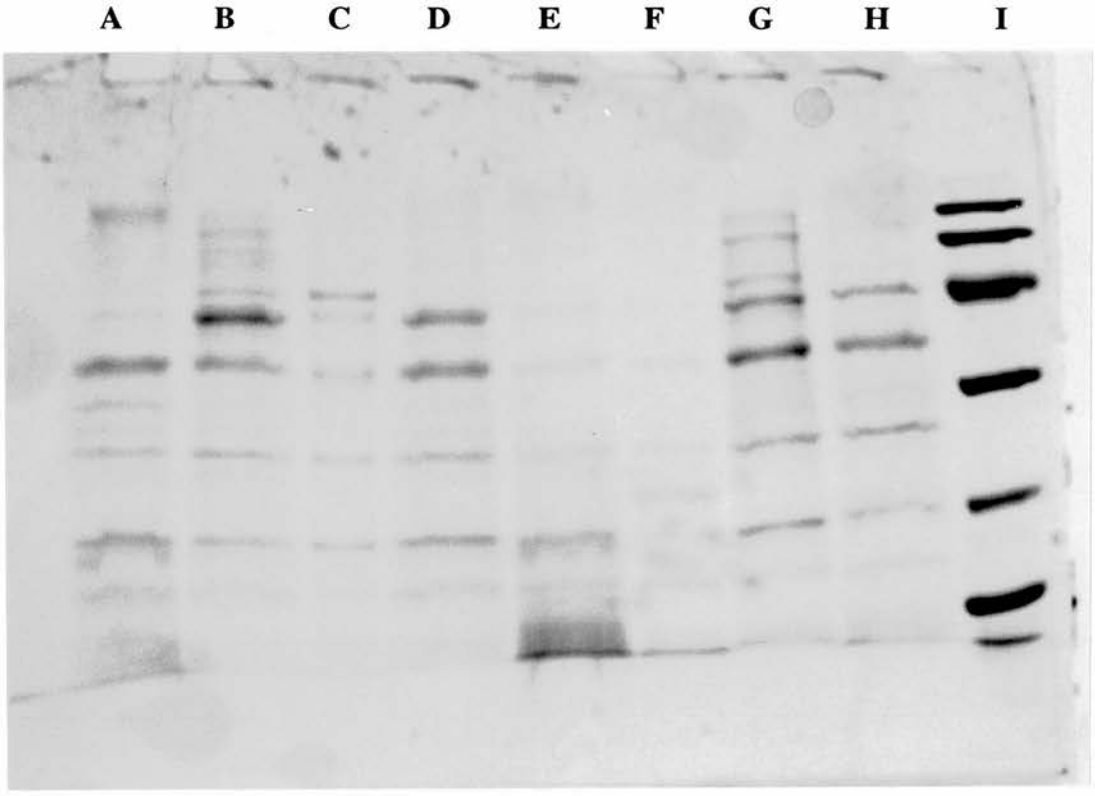


Fig 3.6. OMP Profiles of Paired Clinical Isolates of *P.aeruginosa*.

Tracks are as follows:- Lane A, molecular mass standards: Lane B, parent strain 91-28: Lane C, resistant isolate of 91-28, 91-36: Lane D, 90-62: Lane E, resistant isolate of 90-62, 90-67: Lane F, 8252: Lane G, resistant isolate of 8252, 3221: Lane H, C48: Lane I, C48 grown in the presence of ciprofloxacin: Lane J, strain C48s which reverted to sensitivity during passage experiments.

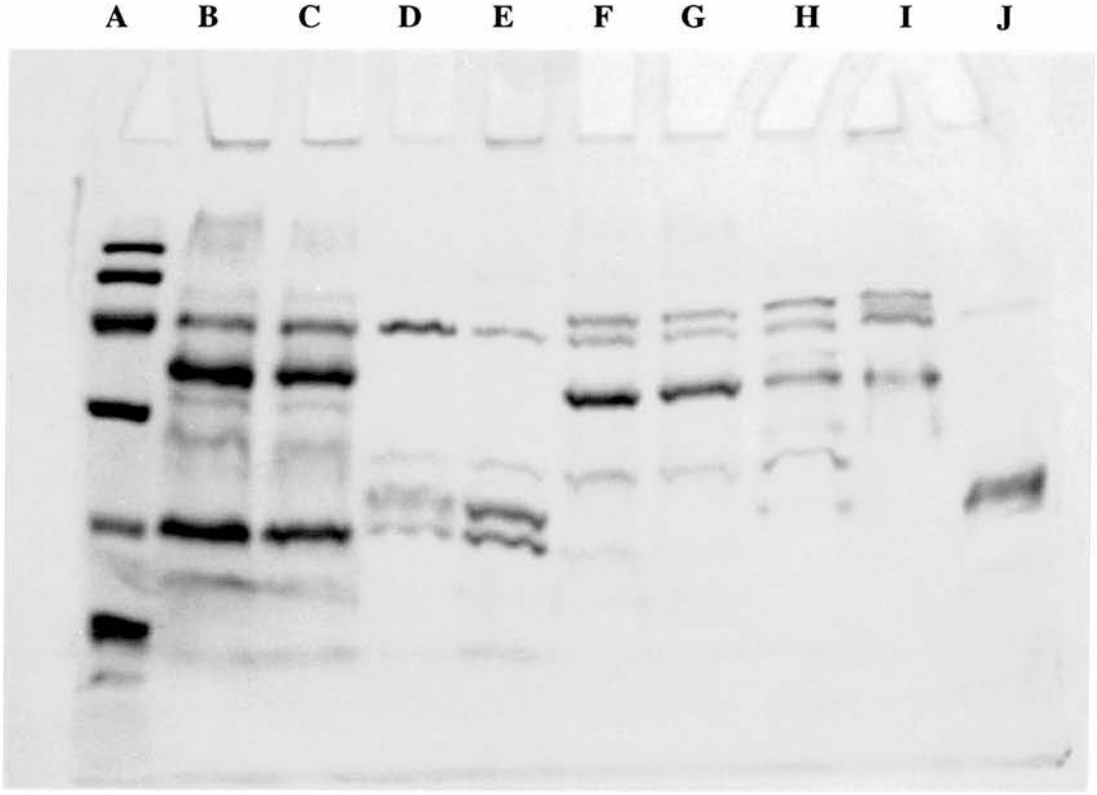


Fig 3.7. OMP Profiles of the 91-32 Series of Paired Clinical Isolates.

Tracks are as follows:- Lane A, the sensitive parent strain 91-32: Lane B, the ciprofloxacin-resistant isolate 91-33: Lane C, 91-41: Lane D, 91-40: Lane E, the ceftazidime-resistant isolate 91-42: Lane F, 91-44: Lane G, 91-46: Lane H, 91-76: Lane I, molecular mass standards.

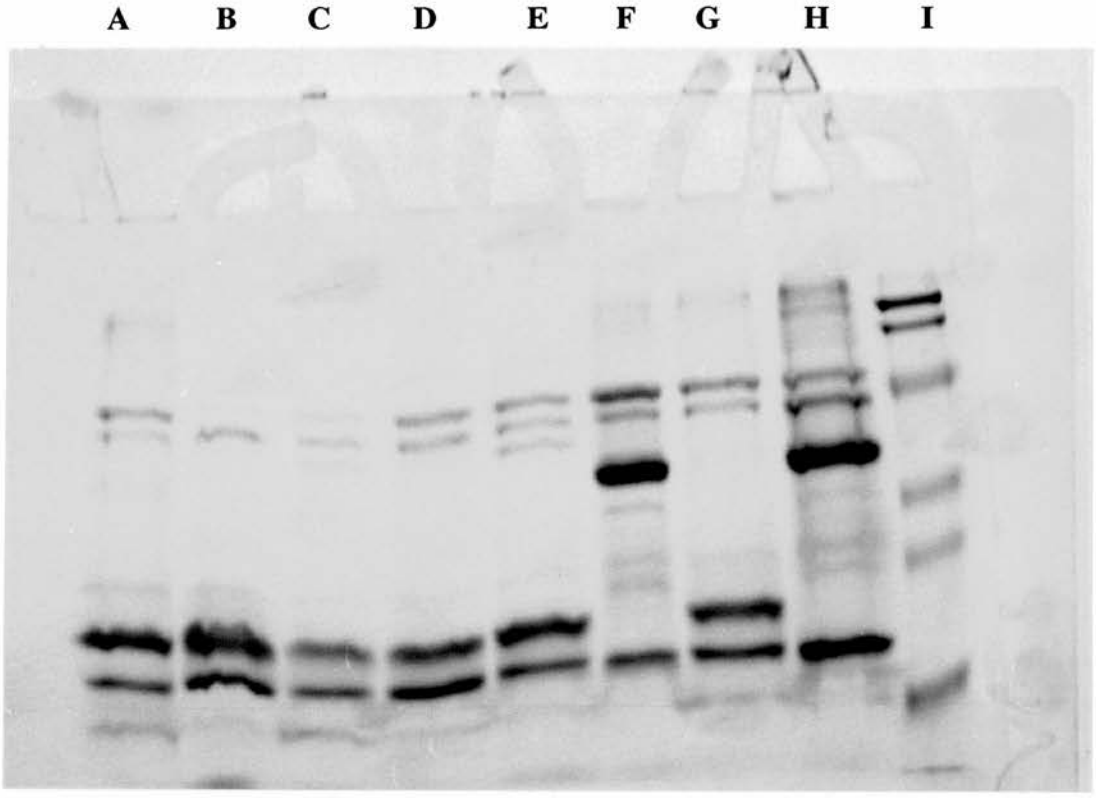
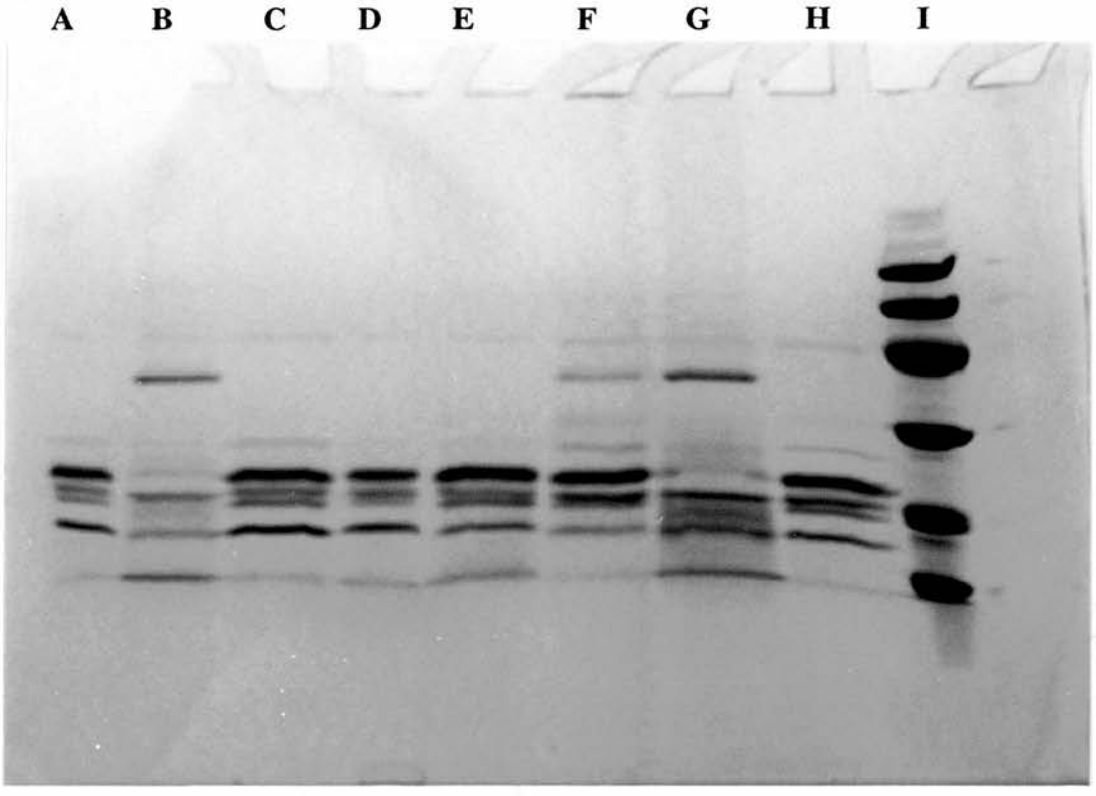


Fig 3.8. OMP Profiles of Porin Preparations of the 91-32 Series of Paired Clinical Isolates.

Tracks are as follows:- Lane A, the parent strain 91-32: Lane B, 91-33: Lane C, 91-41: Lane D, 91-40: Lane E, 91-42: Lane F, 91-44: Lane G, 91-46: Lane H, 91-76: Lane I, molecular mass standards.



To confirm that the 36kD protein was responsible for ciprofloxacin resistance, the parent strain 91-32 was plated onto media containing twice the MIC of ciprofloxacin and mutants selected. The mutational frequency was 2.1 in 10^6, and the MICs of quinolones, β-lactams, gentamicin, imipenem and carbenicillin were determined for the resulting mutants (Table 3.12). In all cases ofloxacin resistance increased with ciprofloxacin resistance, but not to the same extent, as isolates 91-32-8, 91-32-9 and 91-32-11 showed a two to four-fold increase in ofloxacin resistance compared to the rest of the mutant isolates. Also, β-lactam resistance in the mutant strains remained stable, unlike the results observed in their clinical counterparts.

Strains 91-32-1, 91-32-3, 91-32-4, 91-32-6, 91-32-7 and 91-32-10 developed gentamicin resistance, which was not linked to resistance to any other non-quinolone antibiotic. Four strains, 91-32-5, 91-32-8, 91-32-9 and 91-32-11 exhibited hypersusceptibility to gentamicin, coupled with hypersusceptibility to imipenem. These strains, and strain 911-32-3, which was only imipenem hypersusceptible, were the strains observed to have the highest ofloxacin MICs, suggesting that imipenem hypersusceptibility and high levels of ofloxacin resistance might be linked. Strain 91-32-2 was the only imipenem-resistant strain, and strains 91-32-9, 91-32-11 and 91-32-5 exhibited lower carbenicillin MICs than the parent strain.

STRAIN	MIC mg/l						
	CIP	OFX	GN	CAZ	IM	CTX	CAB
91-32	0.25	1	1	2	2	8	32
91-32-1	2	4	8	2	1	16	64
91-32-2	2	8	1	1	4	8	32
91-32-3	2	8	4	2	0.5	8	32
91-32-4	2	4	4	2	2	8	64
91-32-5	4	8	0.25	1	0.5	16	16
91-32-6	2	4	8	1	1	16	64
91-32-7	2	4	10	2	1	>8	>8
91-32-8	4	16	0.25	2	0.25	8	>8
91-32-9	2	16	0.25	1	0.5	8	8
91-32-10	2	4	10	2	1	>8	>8
91-32-11	2	16	0.25	2	0.25	8	8

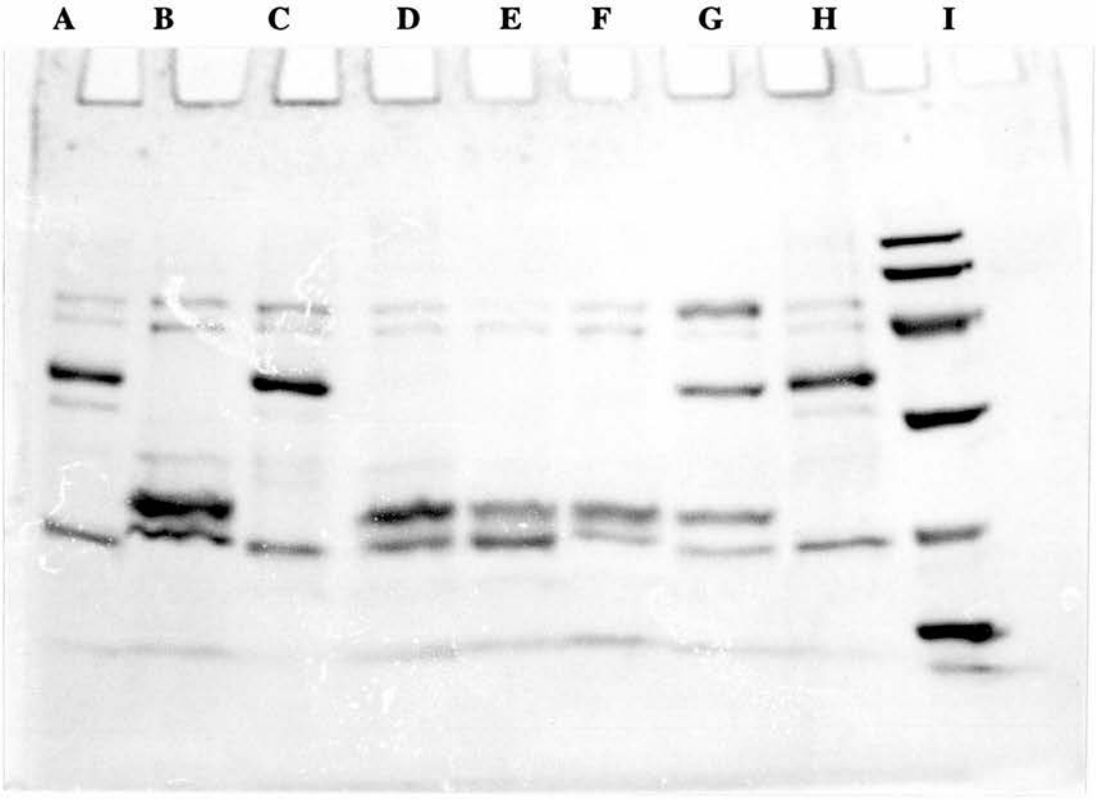
CIP-ciprofloxacin, OFX-ofloxacin, GN-gentamicin, CAZ-ceftazidime, IM-imipenem, CTX-cefotaxime, CAB-carbenicillin.

Table 3.12. MICs of 91-32 Mutant Strains.

The OMP profiles of six selected 91-32 mutants were examined by SDS-PAGE (Fig 3.9) and found to be similar to mutations seen in the series of paired clinical isolates (refer to Fig 3.7). Strains in lanes A, C and H possessed identical profiles to 91-44 and 91-76. Strain 91-32-5 (lane G) also had a similar profile to 91-44, but exhibited an extra 22kD protein and a marked increase in expression of a 51kD protein. Strains 91-32-2 (lane B) and 91-32-4 (lane F) exhibited similar profiles to 91-41 and 91-40.

Fig 3.9. OMP Profiles of Ciprofloxacin-Resistant Laboratory Mutants of 91-32.

Tracks are as follows:- Lane A, mutant strain 91-32-1: Lane B, 91-32-2: Lane C, 91-32-3: Lane D, parent strain 91-32: Lane E, ciprofloxacin-resistant clinical isolate 91-33: Lane F, 91-32-4: Lane G, 91-32-5: Lane H, 91-32-6: Lane I, molecular mass standards.



The idea that high level ofloxacin resistance and imipenem hypersusceptibility could be linked to the production of a 36kD protein was investigated further. Mutants were selected from the ciprofloxacin-resistant strain 91-33 with ofloxacin concentrations of four times the MIC at a frequency of 5.4 in 10^6. Ten strains were then investigated for resistance to ofloxacin, ciprofloxacin and imipenem (Table 3.13), and the OMP profiles of 4 of these strains studied (Fig 3.10).

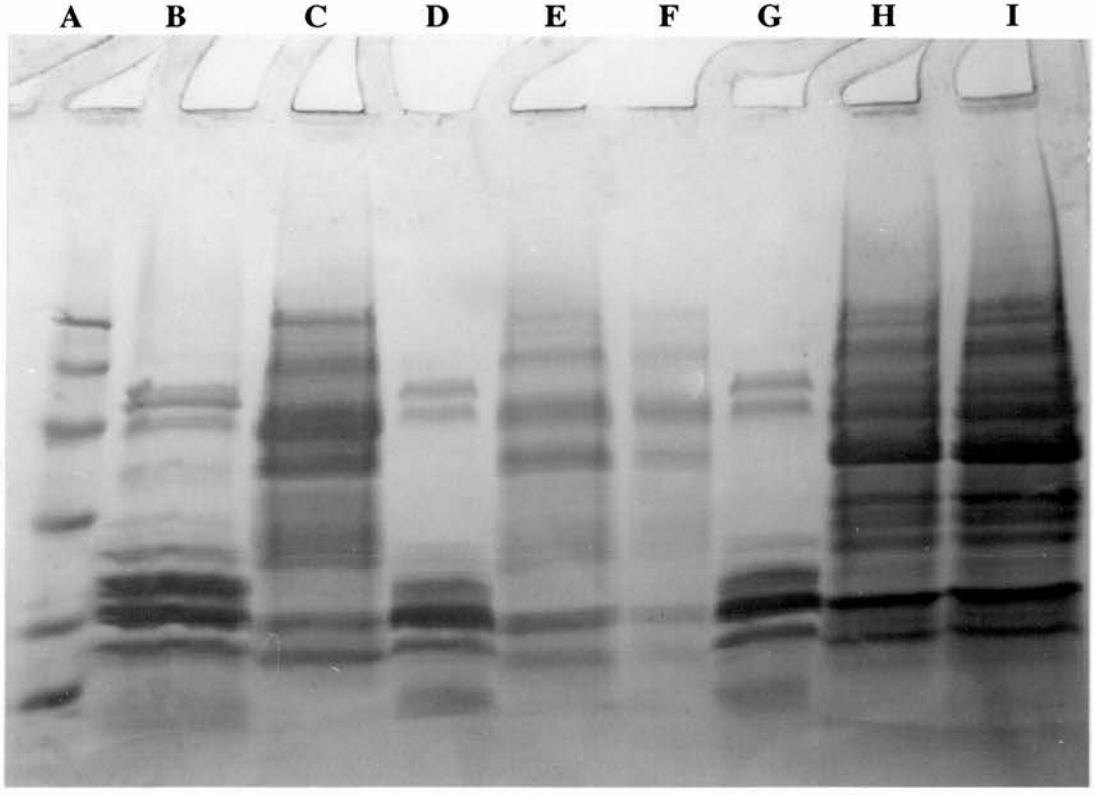
STRAIN	MIC mg/l		
	Ciprofloxacin	Ofloxacin	Imipenem
91-33-1	4	32	1
91-33-2	4	32	1
91-33-3	4	16	2
91-33-4	1	16	2
91-33-7	2	8	2
91-33-12	1	8	2
91-33-14	4	16	2
91-33-17	2	16	2
91-33-21	1	8	2
91-33-22	1	8	2

Table 3.13. MICs of Mutants of 91-33.

In four strains, 91-33-4, 91-33-12, 91-33-21 and 91-33-22 a two-fold alteration in ofloxacin MIC did not affect the ciprofloxacin MIC (Table 3.13). In the remaining strains, increases in ofloxacin resistance brought about increases in ciprofloxacin resistance. Imipenem resistance, however, remained stable. Similar results were obtained on repeating the experiment, suggesting that the imipenem susceptibility observed in previous experiments was not linked to high ofloxacin resistance. Also, as quinolone resistance and the increased production of the 36kD outer membrane protein appear to be linked, alterations in imipenem MICs are probably not associated with altered expression of the 36kD membrane protein.

Fig 3.10. OMP Profiles of Ofloxacin Resistant Laboratory Mutants of 91-33.

Tracks are as follows:- Lane A, molecular mass standards: Lane B, the parent strain of the series of paired isolates, 91-32: Lane C, the ciprofloxacin-resistant laboratory mutant of 91-32, 91-32^{R}: Lane D, strain 91-33: Lane E, ofloxacin-resistant laboratory mutant of 91-33, 91-33-1: Lane F, 91-33-2: Lane G, 91-33: Lane H, 91-33-12: Lane I, 91-33-14.



The OMPs of the 91-33 mutant strains appear to have altered, when compared to the sensitive parent strain, 91-33 (lanes D and G, Fig 3.10). The two resistant strains 91-33-1 (lane E) and 91-33-2 (lane F) have identical OMPs which lack a 51kD protein and have gained a 36kD protein, when compared to the parent strain 91-33 (lanes D and G). The ofloxacin-resistant mutants display a similar profile to the ciprofloxacin-resistant mutant 91-32^{R} (lane C). The strain 91-32^{R} is a ciprofloxacin-resistant laboratory strain produced from the original parent strain of the series of paired isolates, 91-32. These results suggest that the proteins at 51kD and 36kD are responsible for quinolone resistance. The strains 91-33-12 and 91-33-14, in lanes H and I respectively, have identical OMP profiles which exhibit extra protein bands at 50kD, 36kD and 32kD, in comparison with their parent strain 91-33.

It was attempted to confirm that the production of the 36kD protein was linked to quinolone resistance by reverting to sensitivity the ciprofloxacin-resistant strain 91-33. Strain 91-33 was grown on media containing 1/2 MIC values of ciprofloxacin and replica-plated onto drug-free PIA. Strain 91-33 was also seeded onto drug-free PIA and passaged daily, as described in Methods 2.6.1 previously, for 15 days. In all experiments no reversion to sensitivity occurred, suggesting that resistance was stable and, not surprisingly, no alterations in OMPs were identified.

3.3.2 Indirect Analysis of OMP Mutations With EDTA.

Ethylene diamine tetraacetic acid (EDTA) is a chelating agent which is responsible for disrupting bacterial cell outer membranes. Isolates with OMP permeability mutations may be detected by performing MICs in the presence and absence of EDTA (Sato *et al* 1986; Amyes and Smith 1977). In order to determine the most effective concentration at which EDTA should be used, MICs to EDTA were carried out on a selection of 14 strains on IST agar (Table 3.14).

STRAIN	EDTA CONCENTRATION (mM)										
	0	0.1	0.25	0.5	1	2.5	5	10	25	50	100
PAO1	+	+	+	+	+	+	+	-	-	-	-
U423	+	+	+	+	+	+	-	-	-	-	-
C32	+	+	+	+	+	+	+	+/-	-	-	-
C49	+	+	+	+	+	+	+	+/-	-	-	-
3	+	+	+	+	+	+	+	+/-	-	-	-
271	+	+	+	+	+	+	-	-	-	-	-
4125	+	+	+	+	+	+	+	+/-	-	-	-
4133	+	+	+	+	+	+	+	-	-	-	
4158	+	+	+	+	+	+	-	-	-	-	-
4161	+	+	+	+	+	+	-	-	-	-	-
4269	+	+	+	+	+	+	+	+/-	-	-	-
4352	+	+	+	+	+	+	+	+/-	-	-	-
4374	+	+	+	+	+	+	+	-	-	-	-
4495	+	+	+	+	+	+	-	-	-	-	-

+ indicates growth, - indicates no growth, +/- indicates barely visible growth.

Table 3.14. MICs of EDTA for Clinical Isolates of *P.aeruginosa*.

The table demonstrates that EDTA has different effects on *P.aeruginosa* strains. All strains were unaffected by EDTA concentrations below 2.5mM. Growth of resistant strains 271, 4158, 4161 and 4495 was inhibited at an EDTA concentration of 5mM, as was growth of a sensitive strain U423. Growth of another sensitive strain, PAO1, was inhibited at 10mM EDTA, as was growth of the resistant strains 4133 and 4374. Strains C32, C49, 3, 4125, 4269 and 4352 demonstrated barely visible growth at EDTA concentrations of 10mM. The table also demonstrates that EDTA is most effective in a range between 2.5mM and 10mM, and that it completely inhibits bacterial growth at concentrations over 10mM.

The effects of EDTA on ciprofloxacin resistance were investigated for six sensitive isolates and 25 resistant isolates by a geometrical progression chequerboard technique on IST agar. (Table 3.15). The EDTA concentration range chosen was between 1 and 10mM, as this had previously been shown to damage bacterial cell membranes without preventing cell growth (refer to Table 3.14).

STRAIN	MIC (mg/l) to ciprofloxacin at EDTA concentration of :-				
	0mM	1mM	2mM	4mM	8mM
PAO1	0.25	0.25	0.125	0.125	-
U423	0.125	0.125	0.125	0.125	-
C1	16	8	4	2	-
C2	4	2	2	0.5	0.125
C32	4	2	1	1	-
C48	8	8	4	0.25	-
C48s	0.125	0.125	0.125	0.125	-
C49	2	1	0.25	0.125	-
3	>32	32	16	0.5	-
52	1	0.5	0.5	0.25	-
271	4	2	2	1	-
273	8	4	2	1	-
274	4	4	1	1	-
4124	32	16	4	4	0.25
4125	32	16	8	4	0.25
4131	16	8	4	4	0.25
4133	32	16	16	8	0.25
4149	8	4	1	1	0.125
4158	8	2	1	0.5	0.125
4161	8	8	4	1	-
4269	16	8	4	2	0.125
4352	8	4	1	0.125	-
4374	8	4	1	0.125	-
4375	4	2	1	1	-
4495	8	4	4	1	-
4532	8	4	2	0.5	-
5762	0.25	0.125	0.125	0.125	0.125
6397	0.25	0.125	0.125	0.125	0.125
90-62	2	1	0.5	0.5	0.25
91-32	0.25	0.125	0.125	0.125	-
91-33	1	0.5	0.25	0.25	-

Table 3.15. MICs for Clinical Isolates of *P.aeruginosa* to Ciprofloxacin in the Presence of EDTA.

On studying the results, the growth of 64.5% of the strains was inhibited at an EDTA concentration of 8mM, and of the remaining strains only 4124, 4125, 4131, 4133 and 90-62 grew on plates above the lowest ciprofloxacin concentration of 0.125mg/l. This indicated that the EDTA was interfering with bacterial growth; and so the 8mM EDTA results were ignored for the purpose of interpreting the data.

The presence of EDTA in the medium appeared to exert little or no effect upon the MICs of the quinolone-sensitive isolates PAO1, U423, C48s, 5762, 6397 and 91-32. On considering the resistant isolates, most strains, with the exception of C48, 274, and 4161, exhibited a two-fold decrease in the ciprofloxacin MIC. Strain 4158, however, exhibited a four-fold decrease in ciprofloxacin MIC, suggesting that it might possess a membrane alteration which contributed to quinolone resistance.

At a concentration of 2mM EDTA many strains exhibited a significant decrease in ciprofloxacin MICs, with the previously resistant strains C48 and 4161 showing a two-fold decrease in MIC, and 274 a four-fold decrease. Strains C32, C49, 274, 4149, 4158, 4352, 4374 and 4375 had become ciprofloxacin-sensitive. These strains had initially demonstrated low to moderate quinolone resistance. Strains C2, 271 and 4495 were unaffected by an increase in EDTA concentration.

Most strains were observed to have become ciprofloxacin-sensitive in the presence of 4mM EDTA concentrations. Strains C1 and 4269 exhibited an MIC of 2mg/l which is the break point for resistance to ciprofloxacin. Strains 4124, 4125, 4131 and 4133 were still moderately ciprofloxacin-resistant. Of these, 4133 was affected the least, indicating that it was likely to possess other quinolone resistance mechanisms coupled with permeability mutations. The strain 3 showed a large decrease in ciprofloxacin MIC between the 2mM and 4mM EDTA concentrations. All strains were observed to be ciprofloxacin-sensitive in the presence of 8mM EDTA.

These results suggest that all of the ciprofloxacin-resistant clinical isolates possess permeability mutations, as disrupting the outer membrane with a chelating agent has decreased their respective MICs of ciprofloxacin. As the strains are affected to varying degrees, however, it is likely that several different permeability mutations exist. The strains exhibiting similar alterations in ciprofloxacin MICs are also likely to possess similar OMP alterations.

To investigate whether disrupting the cell membrane has the same effect on all quinolones, the MICs of 23 strains to ofloxacin and norfloxacin were examined in the presence of 4mM EDTA (Table 3.16). As with ciprofloxacin, the presence of 4mM EDTA lowered the quinolone MIC in all strains except the sensitive strains PAO1 and U423. Again, strains were affected to varying degrees (refer to Table 3.15), although four distinct patterns seemed to emerge. Similar quinolone ratios were exhibited in 48% of the isolates and the sensitive strain U423.

Several strains, C1, 271, 274 and 4149 demonstrated a greater alteration in norfloxacin ratios than in the ratios of the other quinolones, ciprofloxacin and ofloxacin (Table 3.16); which exhibited similar falls in MIC. The ofloxacin ratios in strains C32, 273, 4124, 4352 and 4374 seemed to be less affected by the presence of 4mM EDTA than the other two quinolones, being on average four-fold different.

STRAIN	Initial MIC (mg/l)			MIC mg/l at 4mM EDTA			Ratio of initial MIC to MIC at 4mM EDTA		
	CPX	OFX	NFX	CPX	OFX	NFX	CPX	OFX	NFX
PAO1	0.5	1	2	0.125	1	1	4	1	2
U423	0.25	1	-	0.125	0.5	0.5	2	2	-
C1	16	64	>128	2	8	8	8	8	>32
C2	32	32	64	0.5	8	4	64	4	16
C32	8	32	64	1	8	4	8	4	16
C48	32	64	>128	0.25	16	16	128	4	>4
C49	8	8	32	0.125	1	2	64	8	16
271	8	64	128	1	8	4	8	8	32
273	8	64	64	1	16	4	8	4	16
274	4	8	32	1	2	2	4	4	16
4124	64	128	>128	4	32	32	16	4	>4
4125	64	>128	>128	4	16	32	16	>8	>4
4131	16	64	>128	4	32	64	4	2	>2
4133	64	128	>128	8	32	32	8	4	>4
4149	8	32	128	1	4	4	8	8	32
4158	16	32	64	0.5	2	2	32	16	32
4161	16	64	128	1	8	8	16	8	16
4269	16	128	128	1	8	8	8	16	16
4352	16	32	128	0.125	2	2	128	16	64
4374	16	64	128	0.125	2	2	128	32	64
4375	8	64	128	1	4	8	8	16	16
4495	16	64	128	1	8	8	16	8	16
4532	8	64	>128	0.5	8	16	16	8	>8

CPX - ciprofloxacin, OFX - ofloxacin, NFX - norfloxacin.

Table 3.16. Alterations in Quinolone MICs in the Presence of EDTA.

The fourth pattern was demonstrated by strains C48 and C49, where ciprofloxacin was the quinolone most affected by the presence of 4mM EDTA. One strain, C2, exhibited different ratios for all of the quinolones tested. These results suggest that there might be four different permeability mutations exhibited in these strains; the alteration in quinolone ratio being indicative of a particular permeability change. The results also suggest that the different quinolones may be affected by varying degrees when permeability changes occur.

3.4 INVESTIGATION INTO DNA GYRASE MUTATIONS.

Previous work has demonstrated that *P.aeruginosa* strains with high quinolone resistance exhibit mutations in the α subunit of DNA gyrase (Inoue *et al* 1987, Hirai *et al* 1987). This was investigated both directly and indirectly by a four-fold approach. The direct approaches involved 1) isolating the DNA gyrase enzyme and determining its ability to supercoil relaxed pBR322 in the presence of various antimicrobial agents, and 2) amplifying the DNA *gyrA* gene by Polymerisation Chain Reaction (PCR), and sequencing the product by either transformation or direct sequencing methods.

The indirect approaches involved 3) amplifying the DNA gyrase *gyrA* gene by PCR and cleaving the sequence around one of the sites involved in quinolone resistance with the *SstII* restriction enzyme. The final indirect approach 4) involved probing for *gyrA* mutants with a plasmid containing the sensitive *E.coli* wild type *gyrA* gene.

3.4.1 Determination of IC_{50}.

To determine the IC_{50} of DNA gyrase, the enzyme was isolated on a novobiocin/sepharose column and purified by dialysis. DNA gyrase is responsible for supercoiling DNA, and in order to assess its activity, its ability to supercoil relaxed pBR322 was investigated. DNA gyrase was assayed by gel electrophoresis in the presence of doubling dilutions of ciprofloxacin, ofloxacin, norfloxacin and novobiocin. Alterations in the α subunit of DNA gyrase would be reflected by high IC_{50} values for the quinolone antimicrobial agents, and alterations in the β subunit by high IC_{50} values for novobiocin.

The activity of DNA gyrase was established by determining the IC_{50} values of each antimicrobial agent from the agarose gel. Gels were stained with ethidium bromide, visualised under an ultra-violet light source and photographed. The IC_{50} value was calculated from the negative, at the point where the action of the antimicrobial agent inhibited 50% of the supercoiling of the pBR322, by a densitometer (Figs 3.11, 3.12, 3.13). The IC_{50}s of 23 ciprofloxacin-resistant strains and one sensitive, reference strain PAO1 were calculated, and compared, by agarose gel electrophoresis (Table 3.17) (data shown in Appendix ii).

Figure 3.11 demonstrates the IC_{50} of a highly resistant DNA gyrase from strain C32. Figure 3.12 shows the inhibition of pBR322 supercoiling in the presence of a moderately resistant DNA gyrase, from the strain 4161. Figure 3.13 demonstrates the supercoiling ability of a DNA gyrase which is only slightly resistant to 4-quinolones, and was isolated from strain 4532.

Fig 3.11. IC_{50} of Strain C32.

Tracks are as follows:-

Block A, the first track contains no drug and subsequent tracks contain sequential increases in ciprofloxacin dilutions from 2 to 256mg/l: Block B, the first track contains no drug and subsequent tracks contain sequential increases in ofloxacin dilutions from 2 to 256mg/l: Block C, the first track contains no drug and subsequent tracks contain sequential increases in norfloxacin dilutions from 2 to 256mg/l.

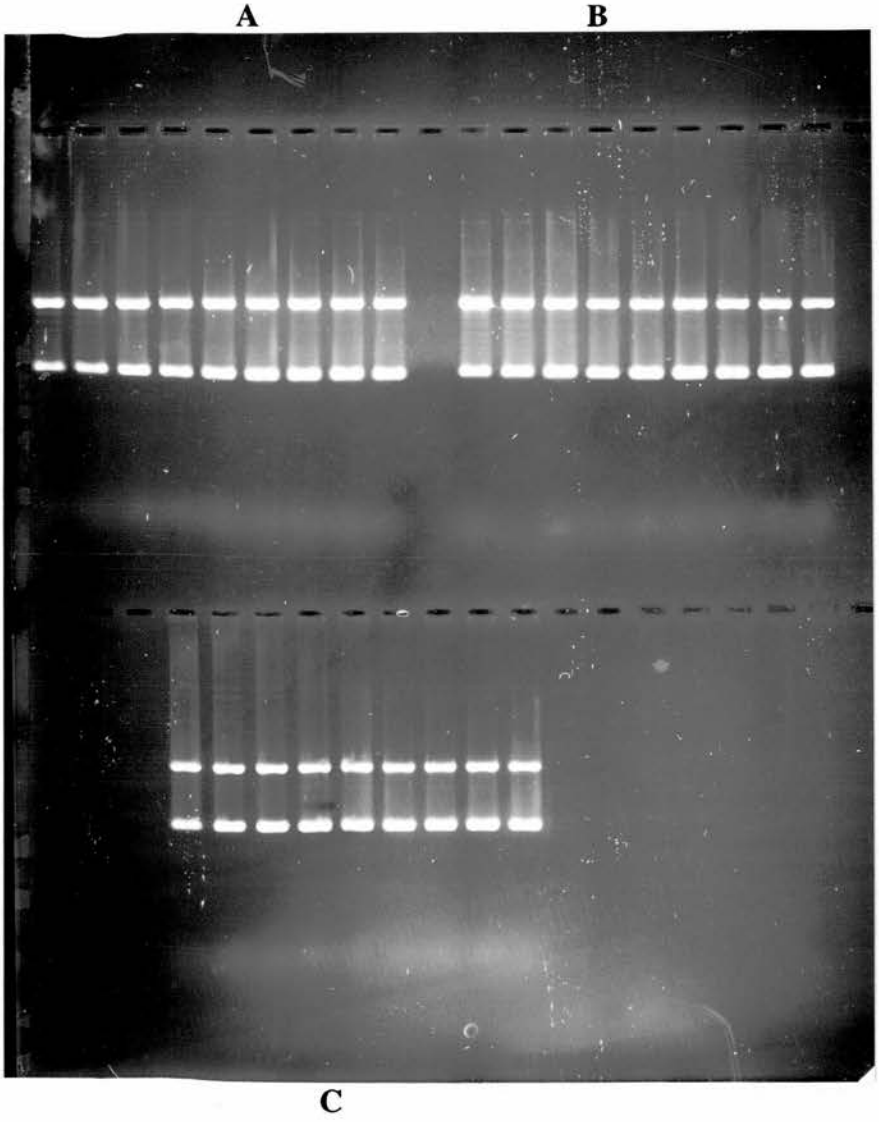


Fig 3.12. IC_{50} of Strain 4161.

Tracks are as follows:- Block A, the first track contains no drug and subsequent tracks contain sequential increases in ciprofloxacin dilutions from 4 to 256mg/l: Block B, the first track contains no drug and subsequent tracks contain sequential increases in ofloxacin dilutions from 4 to 256mg/l: Block C, the first track contains no drug and subsequent tracks contain sequential increases in norfloxacin dilutions from 4 to 256mg/l: Block D, the first track contains no drug and subsequent tracks contain sequential increases in novobiocin dilutions from 0.25 to 8mg/l.

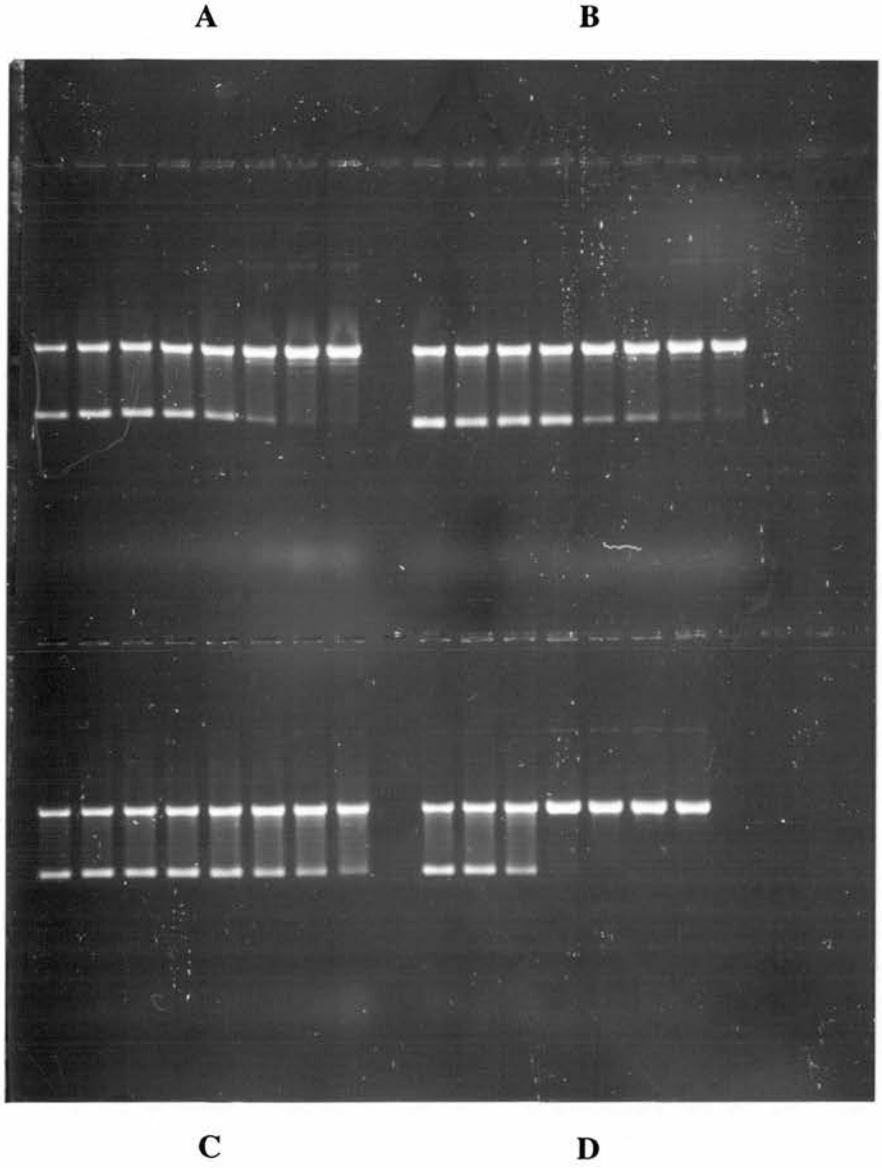
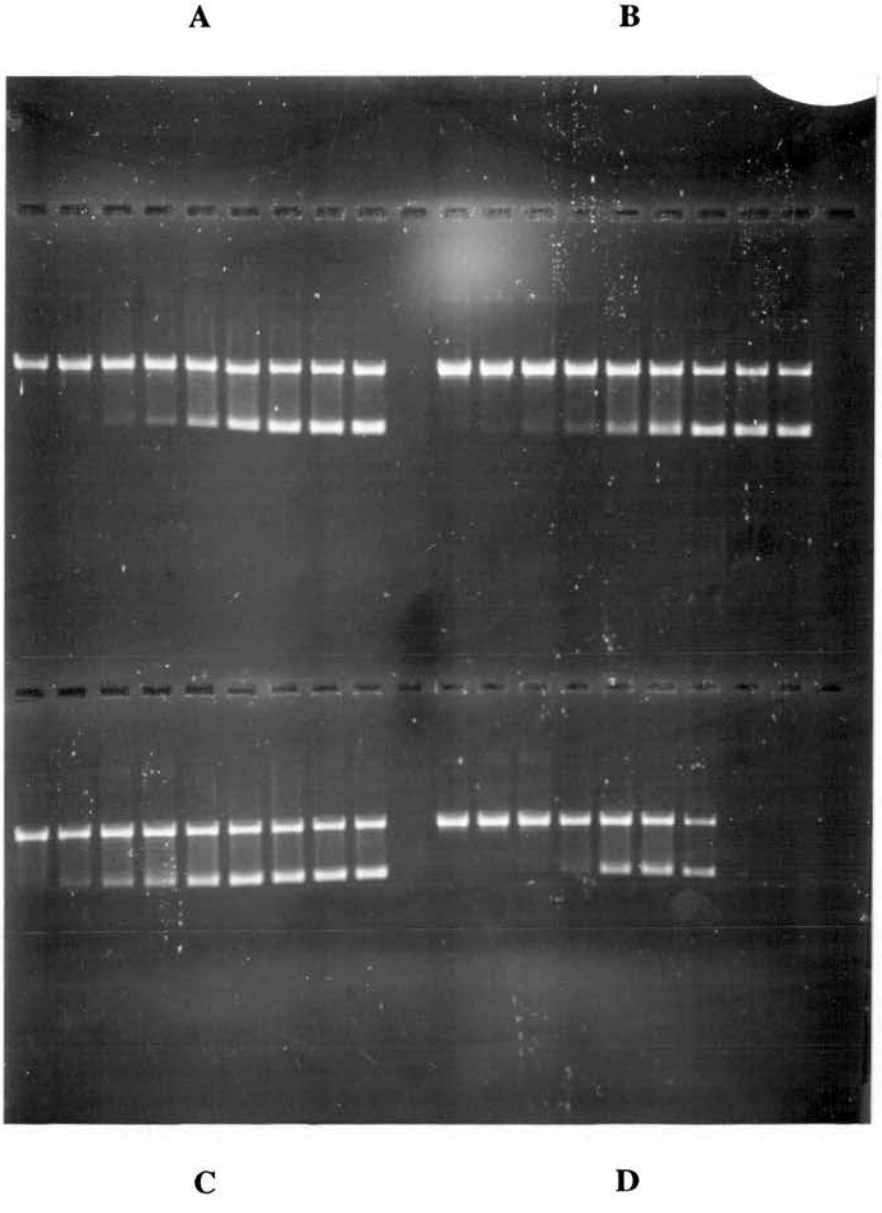


Fig 3.13. IC_{50} of Strain 4532.

Tracks are as follows:- Block A, the first 8 tracks contain sequential ciprofloxacin dilutions from 256 to 2mg/l, and the last track contains no drug: Block B, the first 8 tracks contain sequential ofloxacin dilutions from 256 to 2mg/l, and the last track contains no drug: Block C, the first 8 tracks contain sequential norfloxacin dilutions from 256 to 2mg/l, and the last track contains no drug: Block D, the first 6 tracks contain sequential novobiocin dilutions from 8 to 0.25mg/l, and the last track is drug free.



STRAIN	IC_{50} value mg/l				Ratio Drug / Cip	
	CIP	OFX	NFX	NOV	OFX	NFX
PAO1	0.38	0.85	0.64	0.55	2.24	1.68
C2	10.7	14.1	44.2	0.83	1.32	4.14
C48	19.0	27.3	63.2	0.24	1.44	3.33
273	11.0	17.1	24.9	0.82	1.55	2.26
274	7.7	10.9	20.0	0.25	1.41	2.60
271	16.9	15.2	24.3	0.46	0.9	1.44
4131	5.7	10.6	13.6	-	1.86	2.39
4495	16.1	6.7	15.9	0.35	0.42	0.99
4149	9.9	10.0	23.0	1.27	1.01	2.32
4532	18.1	20.1	56.5	0.8	1.12	3.11
4125	29.0	77.8	67.2	0.51	2.68	2.32
4269	23.9	46.7	25.8	0.14	1.95	1.08
4352	27.2	31.7	53.3	1.12	1.17	1.96
4124	36.32	44.6	108.5	0.25	1.23	2.99
4133	44.3	24.0	43.23	0.47	0.54	0.98
4375	88.3	171.7	185.7	2.0	1.94	2.10
4158	77.9	56.8	172.4	0.89	0.73	2.21
4161	52.2	27.8	200.7	0.71	0.53	3.84
90-62	55.96	90.40	85.52	-	1.62	1.53
C1	178.0	90.1	223.3	0.49	0.51	1.25
C32	>256	>256	>256	3.59	-	-
C49	248.9	219.1	>256	-	0.88	-
3	180.6	171.3	368.8	3.50	0.95	2.04
4374	219.4	87.4	334.7	1.27	0.40	1.53

CIP - ciprofloxacin, OFX - ofloxacin, NFX - norfloxacin, NOV - novobiocin.

TABLE 3.17. IC_{50} Values of Clinical Isolates of *Pseudomonas aeruginosa*.

All IC_{50} values for clinical isolates were significantly greater than those of the reference strain PAO1, suggesting that all of the strains possessed *gyrA* mutations. Strains C32 and 3 also demonstrated significantly higher novobiocin IC_{50} values than the sensitive strain, or any other strains, indicating that a *gyrB* mutation could be present. Strains were compared on their ability to supercoil relaxed pBR322 in the presence of ciprofloxacin. The clinical strains fell into three main categories; those with relatively low IC_{50} values of 4-20 mg/l ciprofloxacin, those with an intermediate value of 50-100 mg/l ciprofloxacin and those with values greater than 150 mg/l ciprofloxacin.

The majority of clinical strains fell into the group with ciprofloxacin IC_{50} values between 4-20 mg/l. In this group ciprofloxacin was the most active quinolone, followed by ofloxacin and norfloxacin, although the ratios of the IC_{50}s of ofloxacin and norfloxacin to ciprofloxacin were variable. Strain 4495 exhibited an ofloxacin IC_{50} which was considerably lower than that of ciprofloxacin, and strains 271 and 4149 had comparable ofloxacin IC_{50}s to those of ciprofloxacin. Strains 4495 and 4269 also had comparable norfloxacin IC_{50}s to those of ciprofloxacin.

In the group with high ciprofloxacin IC_{50} values of greater than 150 mg/l, all of the strains demonstrated much lower or comparable ofloxacin IC_{50}s to those of ciprofloxacin. Strains C1 and 4374 had similar quinolone ratios, although their novobiocin IC_{50} values were different. IC_{50} ratios in the middle group were variable, with several strains, 4133, 4158 and 4161 possessing low ofloxacin IC_{50} values. Strain 4133 also possessed a low norfloxacin IC_{50} value and exhibited similar quinolone ratios to strain 4495, which was present in the low ciprofloxacin IC_{50} group.

The differences in the quinolone IC_{50} ratios may indicate that different gyrase mutations affect various quinolones differently, and that strains exhibiting similar ratios may possess the same gyrase mutations. An alternative suggestion is that strains in the same IC_{50} groups exhibit similar gyrase mutations.

The relationship of ciprofloxacin MICs to ciprofloxacin IC_{50} values in the clinical isolates was also investigated (Fig 3.14). In general, IC_{50} values for each strain were found to be greater than or equal to the corresponding MIC. No correlation was observed between MIC and IC_{50} values in these strains. The relationship between MICs and IC_{50} values for the other quinolones, ofloxacin and norfloxacin, was also investigated (Figs 3.15, 3.16). Again, no correlation was observed between MIC or IC_{50} values. The three graphs also showed three distinct patterns, indicating that quinolone resistant DNA gyrases were affected to different extents by different 4-quinolone structures.

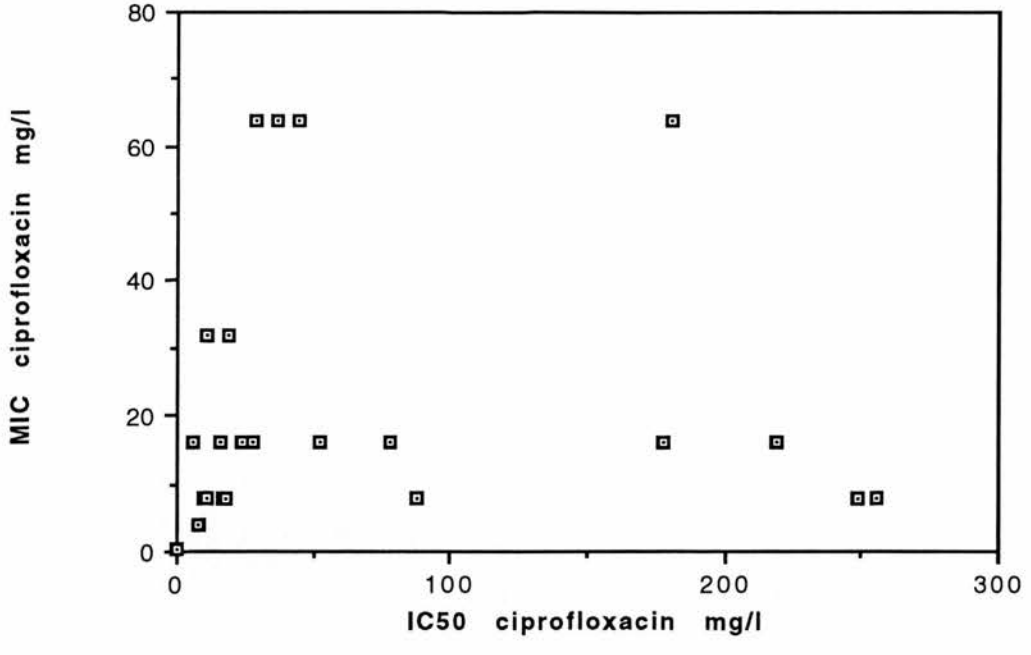


Figure 3.14. Relationship of Ciprofloxacin MIC to IC_{50}

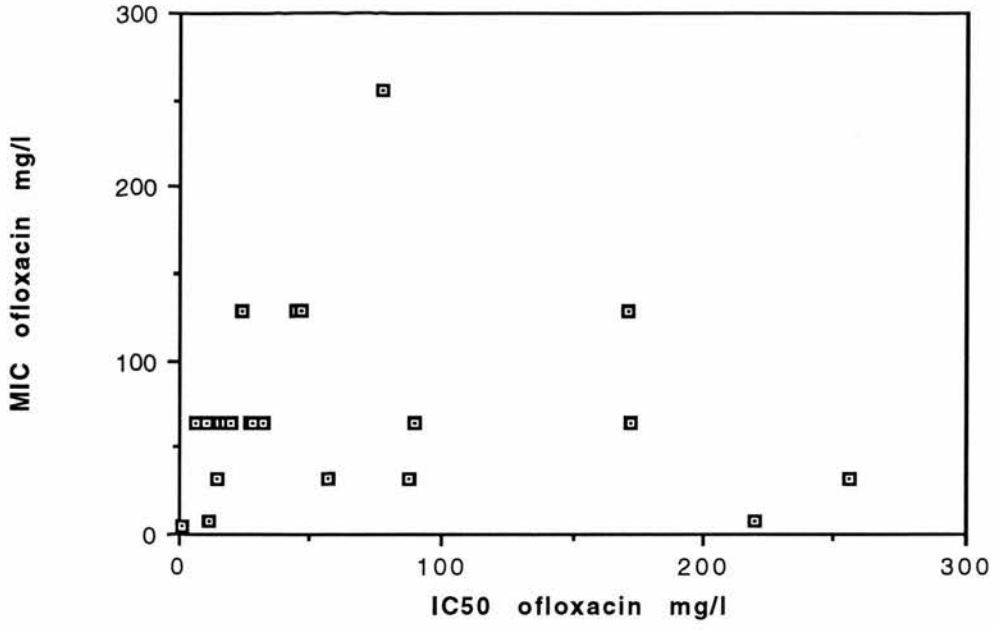


Figure 3.15. Relationship of Ofloxacin MIC to IC_{50}

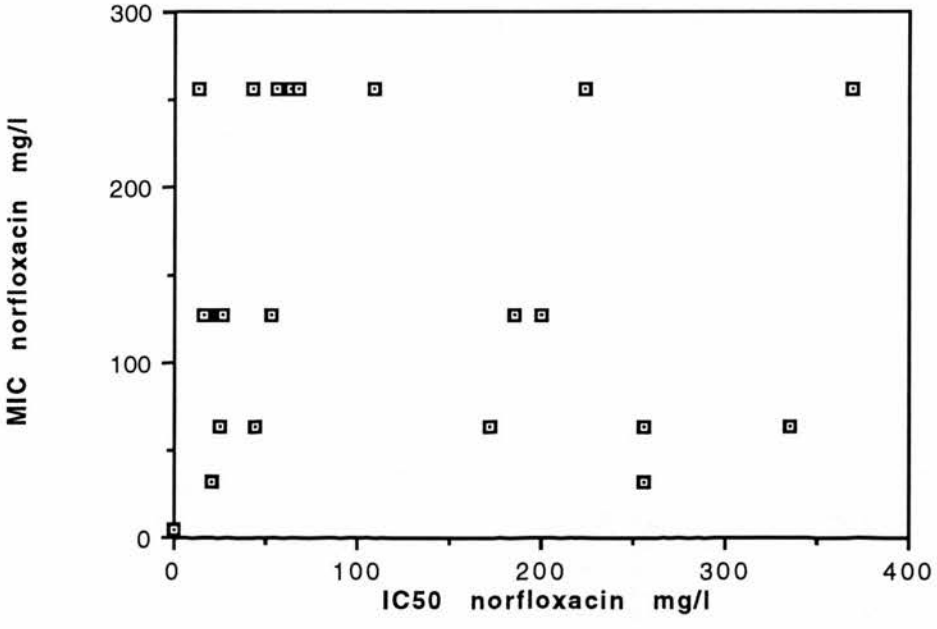


Figure 3.16. Relationship of Norfloxacin MIC to IC_{50}

Direct comparison between the IC_{50} values of each strain is impossible, as each isolate may produce varying amounts of DNA gyrase, which is then used in the assay. To investigate the effect on the IC_{50} of decreasing gyrase levels, the gyrase from a strain with a high ciprofloxacin IC_{50} value, C32, and the gyrase from a strain with moderate ciprofloxacin IC_{50} levels, 90-62, were diluted sequentially. The diluted gyrases were assayed for resistance to ciprofloxacin and the resulting IC_{50}s calculated. In both cases a geometric progression was noticed, by halving the gyrase concentration the IC_{50} value was halved (Fig 3.17, Table 3.18). The dilution of C32 DNA gyrase and its subsequent reduction in IC_{50} levels, however, was insufficient to explain the 100-fold differences between activities of DNA gyrases within the group with low gyrase IC_{50} levels and those with very high gyrase IC_{50} levels.

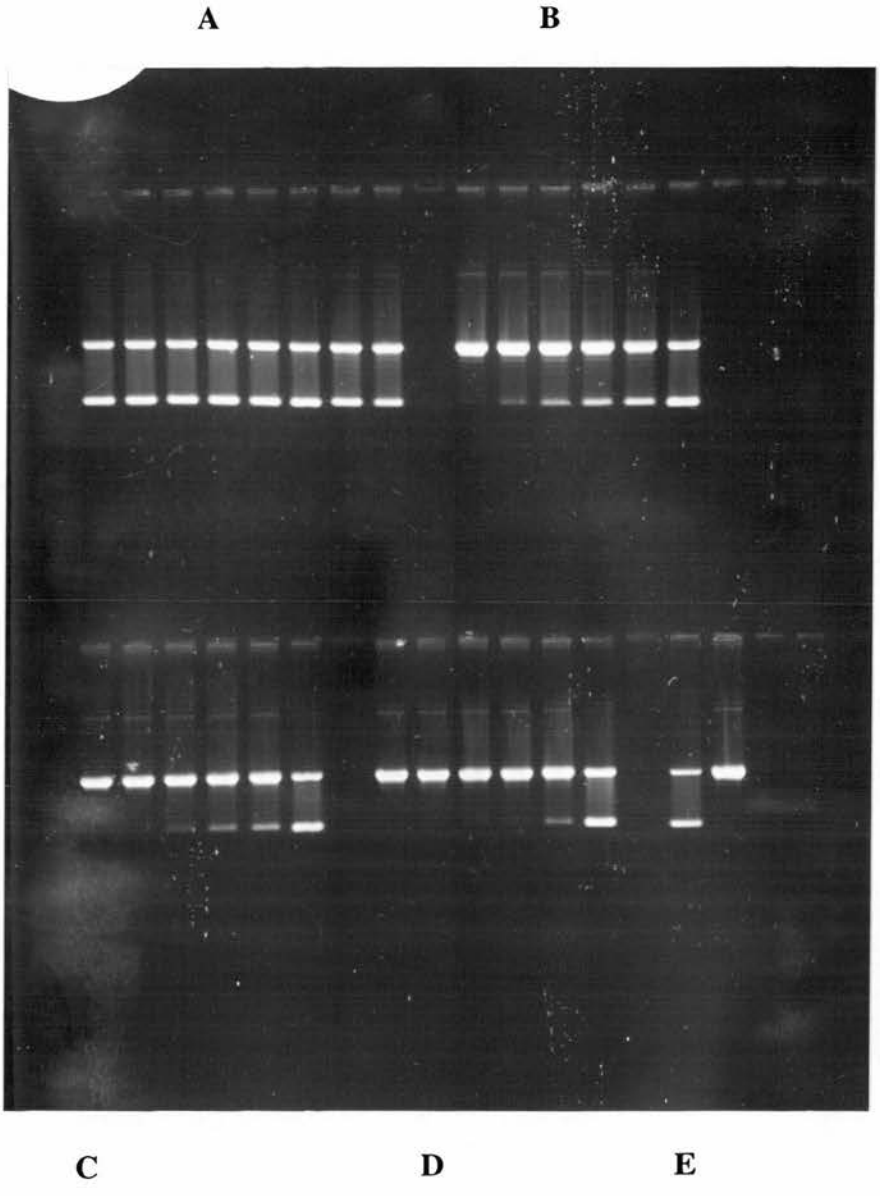
STRAIN	Ciprofloxacin IC_{50} value (mg/l) at dilutions of :-		
	0	50%	75%
C32	112.13	48.83	28.18
90-62	55.96	27.70	-

Table 3.18. Values of Sequential Dilutions of Two DNA Gyrases.

The differences in IC_{50} values within the clinical isolates, the different quinolone ratios, and the differences between MIC and IC_{50} values suggest that there are at least two quinolone resistance mechanisms exhibited by *Pseudomonas aeruginosa*. To investigate the existence of these mechanisms further, a strain in the low IC_{50} group, 4149, and a high IC_{50} group strain, C32, were challenged by plating onto IST agar plates containing ciprofloxacin at twice the MIC of the strains. The two strains were selected as they exhibited the same ciprofloxacin MIC, but were in different IC_{50} groups.

Fig 3.17. IC_{50} of Sequentially Diluted C32

Tracks are as follows:- Block A, sequential gyrase dilutions from 0% to 87.5%: Block B contains undiluted DNA gyrase; the first 5 tracks contain sequential dilutions of ciprofloxacin from 512 to 32mg/l, and the last track contains no drug: Block C contains 50% diluted DNA gyrase; the first 5 tracks contain sequential dilutions of ciprofloxacin from 512 to 32mg/l, and the last track contains no drug: Block D contains 75% diluted DNA gyrase; the first 5 tracks contain sequential dilutions of ciprofloxacin from 512 to 32mg/l, and the last track contains no drug: Block E contains a positive control, pBR322, and a negative control of relaxed pBR322.



The resulting mutants were identified, purified, and then grown in nutrient broth in the presence of quarter MIC values of ciprofloxacin, to maintain the selective pressure. IST agar plates containing ciprofloxacin at twice the new MIC level were then seeded, and mutants isolated and purified as before. The procedure was repeated until mutants of both C32 and 4149 had MICs of ciprofloxacin of 32mg/l. A single mutant colony from each strain was then selected, and their OMPs isolated and examined as described in Methods section 2.7, in order to determine whether membrane protein alterations were responsible for the increase in MIC (Fig 3.18). The DNA gyrases from the mutant strains were also isolated and purified, and their IC_{50} values determined by gel electrophoresis (Table 3.19).

STRAIN	MIC mg/l			IC_{50} mg/l			
	CIP	OFX	NFX	CIP	OFX	NFX	NOV
C32	8	32	64	>256	>256	>256	3.59
$C32^R$	32	128	>128	393.47	398.70	392.49	1.72
4149	8	32	128	9.9	10.0	23.0	1.27
4149^R	32	128	>128	30.0	39.63	62.61	0.49

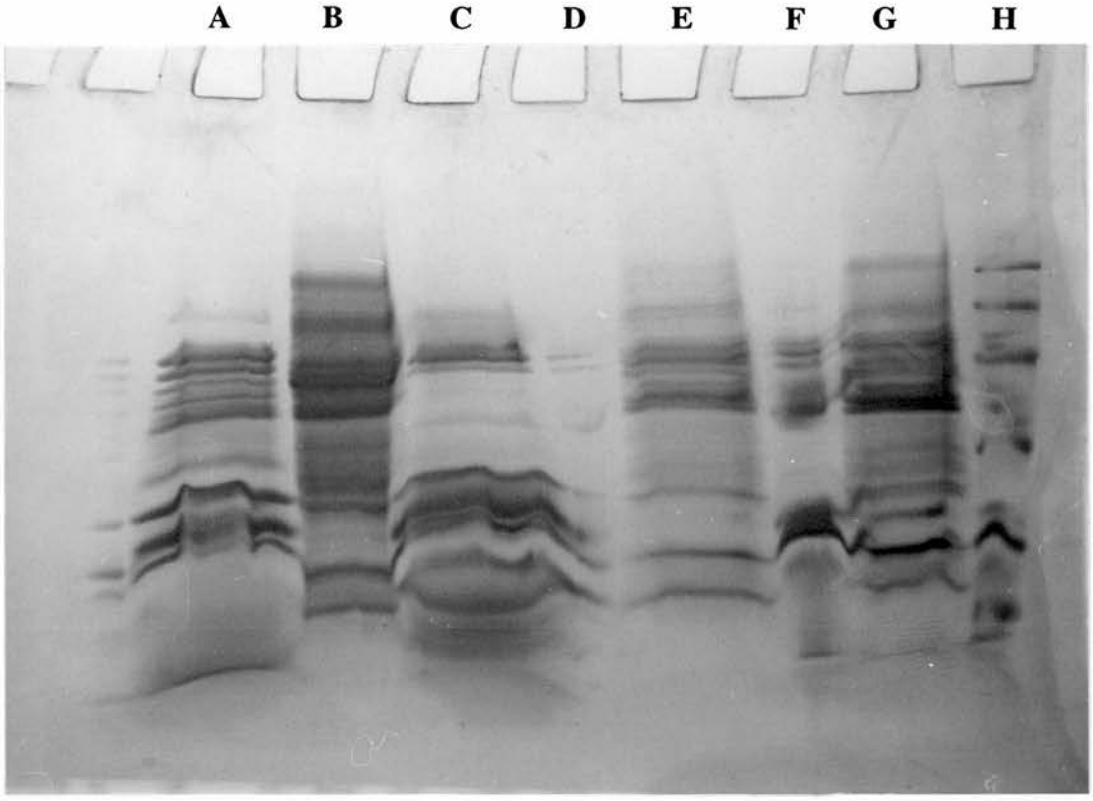
CIP - ciprofloxacin, OFX - ofloxacin, NFX - norfloxacin, NOV - novobiocin.

Table 3.19. The MIC and IC_{50} Values of Mutants of C32 and 4149.

The OMP profiles of C32 and its resistant mutant $C32^R$ appeared to be identical, indicating that the increased resistance was not caused by membrane permeability mutations. The OMP profiles of 4149 and 4149^R were different, with 4149^R exhibiting an extra 38kD protein. This indicates that the increase in quinolone resistance might have been brought about by an alteration in drug permeability.

Fig 3.18. OMP Profiles of Mutants of C32 and 4149.

Tracks are as follows:- Lane A, C48: Lane B, ciprofloxacin-resistant laboratory mutant of the strain C48s, $C48s^R$: Lane C, the sensitive isolate of C48, C48s, obtained from the passage experiments: Lane D, C32: Lane E, ciprofloxacin-resistant laboratory mutant of C32, $C32^R$: Lane F, 4149: Lane G, ciprofloxacin-resistant laboratory mutant of 4149, 4149^R: Lane H, molecular mass standards.



4149^R also exhibited increased IC_{50} values compared to its parent strain, which indicated that a gyrase mutation had also occurred. The IC_{50} values of 4149^R for each drug investigated appeared to increase uniformly with an increase in MIC, when compared to 4149; the new IC_{50} for each drug was three-fold greater than that of the parent strain. The novobiocin IC_{50} was observed to decrease three-fold. The IC_{50} values of ciprofloxacin, ofloxacin and norfloxacin in strain $C32^R$ could not be compared to those of the parent strain, C32, but a two-fold decrease in the novobiocin IC_{50} was seen.

3.4.2 Direct Analysis of *GyrA* Mutations.

The DNA gyrase sequence of the *gyrA* gene of *Pseudomonas aeruginosa* was obtained from Dr. L.E. Bryan (personal communication), and oligonucleotide primers incorporating a *BamH1* restriction site were used to synthesise a DNA sequence of approximately 300 base pairs, by the Polymerase Chain Reaction (PCR) technique (Disney and Dove, 1991). Multiple copies of the fragment were obtained by PCR for five selected strains, PAO1, C32, C49, 4149 and 4158.

PAO1 was selected to confirm the nucleotide sequence of the sensitive gyrase gene. The strains C32, C49 and 4149 were chosen because they possessed the same ciprofloxacin MIC, but C32 and C49 were in the high IC_{50} group and 4149 was in the low one. The strain 4158 was selected as it was present in the moderate IC_{50} group. The PCR fragments were then either sequenced directly, or restricted with *BamH1*, transformed into competent cells and sequenced by gel electrophoresis. The results are shown in Fig 3.19.

Fig 3.19. Sequencing Gel of C32.

Tracks are as follows:- Block A, C32 with a labelling step of five minutes, the lanes were loaded in the order G, A, T, C: Block B, C32 with a labelling step of two minutes, the lanes were loaded in the order G, A, T, C.

Fig 3.19 shows the nucleotide sequence of C32. Clear nucleotide sequences could not be deduced as bands appeared in every lane, and the alterations in labelling times appeared to have no effect. The multiplication of bands is caused by the re-annealing of primers to the DNA strand and has been observed to be a major flaw in this technique (S.K. DuBois personal communication).

3.4.3 Indirect Analysis of *GyrA* Mutations.

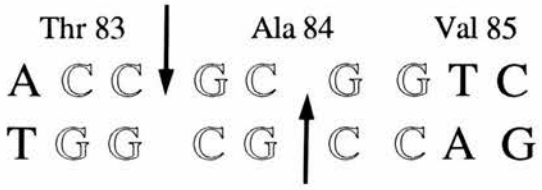
GyrA mutations were analysed indirectly by restricting PCR fragments from 18 ciprofloxacin-resistant clinical isolates with the enzyme *SstII*. *SstII* cleaves the 300 base pair PCR fragment between amino acids 83 and 84 in the sensitive strain PAO1 (Fig 3.20). This site has been reported to be an important active site for quinolone resistance in *E.coli*, and is responsible for high level quinolone resistance. PCR sequences from the clinical isolates which contain a *gyrA* mutation in the codon of either amino acid 83 or 84 will not be cleaved by the enzyme (Table 3.20). Analysis of the PCR fragments in this way will give some indication of the prevalence of mutations at this site. The restricted fragments were then analysed by gel electrophoresis and photographed as described previously (Fig 3.21).

STRAINS CLEAVED BY *SstII* RESTRICTION	STRAINS UNCLEAVED BY *SstII* RESTRICTION
PAO1, 4374, 4532, 4149, 4375, C32, C48, 273, 4133, 4131.	4124, 4125, C1, C2, 271, 274, 4161, 4269

Table 3.20. The Effect of *SstII* Restriction on the Unpaired Ciprofloxacin-Resistant Clinical Isolates.

Table 3.20 indicates that most strains are cleaved by the *SstII* restriction enzyme. These strains do not harbour nucleotide alterations in either amino acids 83 or 84. The strains unaffected by the *SstII* restriction enzyme, however, do contain a mutation in either amino acid 83 or amino acid 84. These results indicate that eight clinical isolates contain at least one mutation in the DNA gyrase gene, which contributes to their overall quinolone resistance.

Fig 3.20. The Cleavage Site of *SstII*.



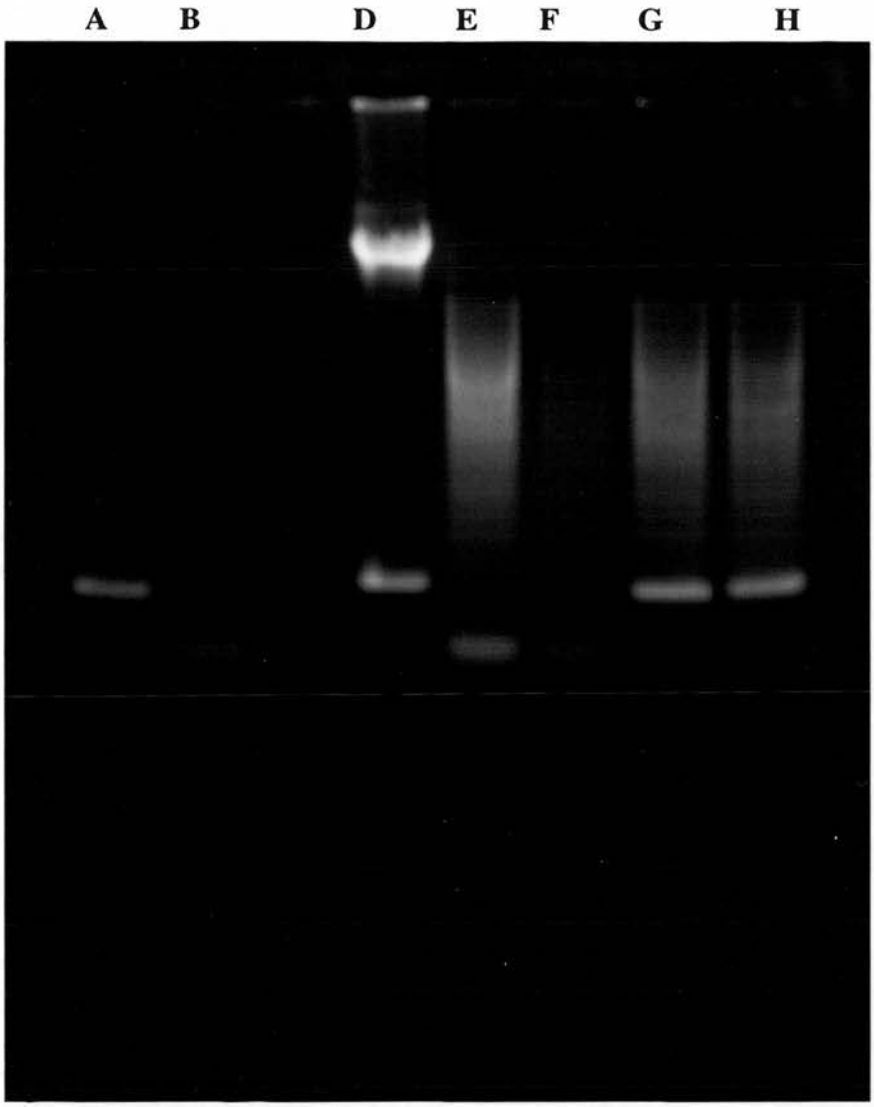
Letters outlined denote the nucleotide sequence recognised by the enzyme.

3.4.4 Probing for *GyrA* Mutations.

To confirm the presence of *gyrA* mutations, the resistant clinical isolates were probed with pNJR3-2, which contained the wild-type *Escherichia coli gyrA* gene. The wild-type gene is quinolone-sensitive and has been shown to be dominant over the quinolone-resistant DNA gyrase. On introducing the plasmid into the quinolone-resistant clinical isolates, the isolates would be expected to revert to quinolone sensitivity, or to become more quinolone-sensitive if they harboured other quinolone resistance mechanisms.

Fig 3.21 Gel of *SstII* Cleaved PCR Fragments.

Tracks are as follows:- Lane A, PAO1 PCR fragment: Lane B, *SstII* restricted PAO1 fragment: Lane D, C32 PCR fragment: Lane E, *SstII* restricted C32 fragment: Lane F, *SstII* restricted C32 fragment: Lane G, *SstII* restricted 4124 fragment: Lane H, 4124 PCR fragment.



The conjugation method of Robillard (1990) was used to introduce the probe into *P.aeruginosa*, but in spite of numerous attempts the plasmid did not appear to have transferred. A major obstacle to the selection of transconjugants was the high intrinsic resistance of *P.aeruginosa* to the selective agents used. pNJR3-2 contained antibiotic resistance markers for both tetracycline and kanamycin; but the recipient *P.aeruginosa* strains also exhibited resistance to these markers, making it impossible to distinguish between recipient strains and transconjugants. An attempt was made to select the transconjugants by replica plating the suspected transconjugant colonies onto PIA plates containing ciprofloxacin at 1/8, 1/4, 1/2 and the MIC value of the relevant strain, but no transconjugant strains were identified.

Alternative conjugation methods were attempted to introduce the plasmid into the *P.aeruginosa* strains. The first was the method of Amyes and Gould (1984) which involved the donor and recipient strains being grown statically in 10ml nutrient broth, overnight at 37°C. Either 1ml of the donor strain was added to 1ml of the recipient strain in 10ml fresh broth, or 0.1ml of the donor strain was added to 1ml of the recipient strain, and the broth incubated statically overnight at 37°C. The resulting bacteria were then seeded onto plates containing the selective agent, as described in Materials and Methods section 2.11.3. Again, no transconjugants were isolated.

To investigate whether the lack of success in transferring the plasmid was caused by the inability of the plasmid to mobilise, it was attempted to introduce pNJR3-2 into the *E.coli* strain J62-2, again following the method of Amyes and Gould (1984). The transconjugants were selected on proline, histidine and tryptophan (PHT) plates containing rifampicin and tetracycline, and a plasmid preparation carried out on them.

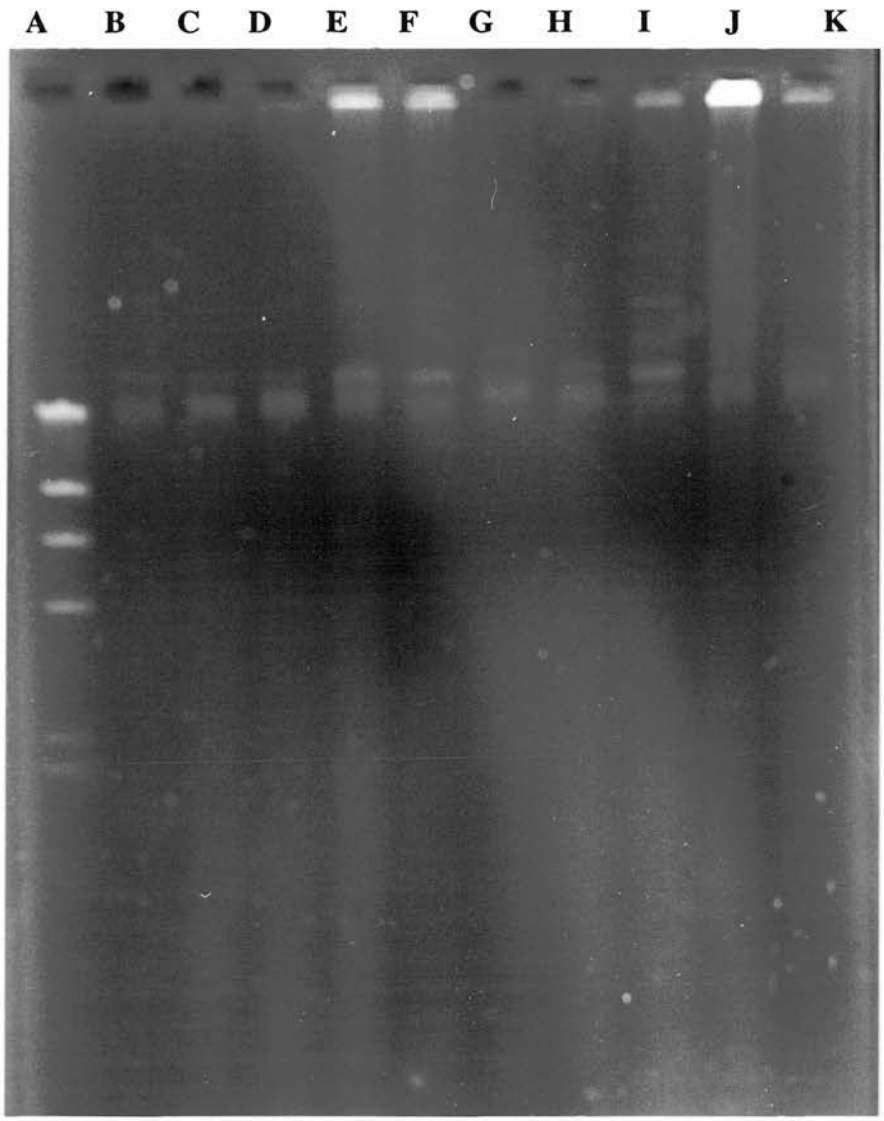
The plasmid preparations were analysed by gel electrophoresis, and provided J62-2 isolates containing the probe (Fig 3.22). This indicated that pNJR3-2 was able to mobilise, and that the transfer problem was probably caused by strain differences between *P.aeruginosa* and *E.coli.*

A triple mating was then carried out in order to introduce the plasmid into a *P.aeruginosa* strain. The *E.coli* strain containing pNJR3-2 was mated with J62-2 as described above, and the J62-2 containing the plasmid was subsequently mated with PAO2, a serine requiring *P.aeruginosa* auxotroph. It was decided to use PAO2 as it was a quinolone-sensitive strain, and its serine requirement enabled recipients to be selected out from donor and transconjugant strains. As before, no transconjugant strains were isolated.

To compensate for the intrinsic resistance to tetracycline exhibited by the *P.aeruginosa* strains, the MICs of the quinolone-resistant strains to tetracycline were determined in the presence of 4mM EDTA (Table 3.21). EDTA was used to disrupt the cell membrane in recipient strains, thus rendering them susceptible to tetracycline and overcoming the intrinsic resistance inherent in the *P.aeruginosa* strains. As the transconjugants would be carrying plasmid-encoded tetracycline resistance, they would be expected to grow at high tetracycline concentrations inthe presence of 4mM EDTA.

Figure 3.22. Plasmid Preparation of J62-2 Demonstrating the Presence of pNJR3-2.

Tracks are as follows:- Lane A, molecular mass standards: Lane B, pNJR3-2: Lanes C - K, transconjugants isolated.



STRAIN	TET resistance (mg/l) in the presence of EDTA concentrations of (mM) :-	
	0	4
PAO1	64	50
C1	128	100
C2	64	100
C32	256	200
C48	>256	200
C49	128	50
3	128	100
271	64	50
273	128	100
274	64	50
4124	128	100
4125	128	100
4131	128	50
4133	128	100
4149	128	100
4158	128	50
4161	128	50
4269	128	100
4352	256	100
4374	256	100
4375	128	100
4495	64	50
4532	128	50

TABLE 3.21. MICs of Tetracycline in the Presence and Absence of EDTA.

The EDTA concentration of 4mM was selected as it had previously been shown to overcome membrane permeability resistance without inhibiting cell growth (refer to Results section 3.3.2). The results demonstrated that most strains retained their tetracycline resistance in the presence of 4mM EDTA, thus indicating that tetracycline resistance in *P.aeruginosa* is likely to be plasmid borne. Strains 4532, 4374, 4352, 4161, 4158, 4131 and C49 did exhibit slight reductions in tetracycline MICs, but even with these strains no transconjugants were obtained.

A final attempt was made to introduce pNJR3-2 into *P.aeruginosa* by electroporation, employing the method of Diver and colleagues (1990a). The plasmid was isolated as described previously (refer to Methods and Materials section 2.11.4) and its purity checked by gel electrophoresis. The plasmid was then introduced into strains PAO1, 4124, 4125 and 3; a control plasmid, pUC18, was also introduced into the four strains. Initial arcing of the electroporation mixture was overcome by diluting both the plasmid and the recipient strain by ten-fold. The solution was then seeded, as before, onto IST agar plates containing tetracycline, but no transconjugants were obtained. Also, pUC18 was not present in any of the resistant transconjugants recovered. The method of Diver and colleagues (1990a) was then modified by increasing the resistivity of the Electrocell Manipulator to 492 ohms, but this produced excessive arcing which could not be overcome by diluting either preparation.

DISCUSSION

The aim of this thesis was to investigate the mechanisms of resistance of *Pseudomonas aeruginosa* to the 4-quinolones by studying the pleiotropic nature of both the outer membrane protein and DNA gyrase mutations. Previous reports had indicated that *P.aeruginosa* exhibits alterations in several OMPs (Hirai *et al* 1987; Robillard and Scarpa 1988; Chamberland *et al* 1989), and reports of DNA gyrase mutations in other bacteria, such as *E.coli, S.aureus* and *B.subtilis* (Hirai *et al* 1986b; Tanaka *et al* 1991; Lampe and Bott 1984) indicated that *P.aeruginosa* might also exhibit several gyrase mutations. No reports, however, had investigated the relationship between MIC and DNA gyrase mutations or DNA gyrase mutations and OMP mutations. It was also unclear whether gyrase mutations developed before or after OMP mutations in quinolone-resistant bacteria. It was hoped, during the course of this study, to address some of these questions.

4.1 MINIMUM INHIBITORY CONCENTRATIONS.

4.1.1 MICs of the Quinolones.

In terms of MICs of the 4-quinolones it was found that the resistant clinical isolates fell into a range of 4-64 mg/l ciprofloxacin, 8-128 mg/l ofloxacin and 32->128 mg/l norfloxacin. These results are similar to MICs reported by many other workers; Robillard and Scarpa (1988) reported quinolone resistances of 4-16 mg/l ciprofloxacin, 8-32 mg/l ofloxacin and 4-32 mg/l norfloxacin and Thomson and colleagues (1991) reported resistances of 4-64 mg/l ciprofloxacin and 4-16 mg/l ofloxacin.

The MICs demonstrated that ciprofloxacin was the most active 4-quinolone, followed by ofloxacin and norfloxacin. Robillard and Scarpa (1988) reported that ciprofloxacin was the most active 4-quinolone, and that ofloxacin and norfloxacin possessed similar activities. Legakis and co-workers (1989) reported that the activity of ciprofloxacin was two- to four-fold higher than that of norfloxacin. More recent reports, however, have indicated that ofloxacin has comparable MICs to ciprofloxacin (Thomson *et al* 1991).

The MICs of the quinolone-resistant clinical isolates exhibited different ratios of ofloxacin and norfloxacin resistance when compared to ciprofloxacin. The sensitive strains PAO1 and U423 demonstrated similar ratios of drug resistance, with ofloxacin 2-4 times less active than ciprofloxacin, and norfloxacin 4-8 times less active than ciprofloxacin. If all of the ciprofloxacin-resistant clinical isolates exhibited identical mutations then the ratios of quinolone MICs to MICs of ciprofloxacin would be expected to be similar, but this was not observed.

The resistant clinical isolates did, however, fall into several similar categories;

1) two strains exhibited lower ciprofloxacin/ofloxacin resistance ratios and ciprofloxacin/norfloxacin resistance ratios than the sensitive strains.

2) four strains exhibited a two-fold increase in ciprofloxacin/ofloxacin resistance ratios and a four-fold increase in ciprofloxacin/norfloxacin resistance ratios, a pattern similar to the sensitive strains.

3) five strains exhibited a four-fold increase in ofloxacin resistance and an eight-fold increase in norfloxacin resistance ratios compared with ciprofloxacin.

4) six strains exhibited a four- to eight-fold increase in ofloxacin resistance ratios and an eight- to sixteen-fold increase in norfloxacin resistance ratios.

The variability in the ratios suggests that the bacteria are responding differently to the selective pressures of the quinolones and also indicates that the isolates are exhibiting several different quinolone mutations. Another conclusion drawn from these results is that different resistance mutations affect various 4-quinolones in different ways. For example, the mutation exhibited by strains 273 and 4269 has the same effect on ofloxacin resistance as norfloxacin resistance, but has less effect on ciprofloxacin resistance, as the ciprofloxacin MIC is eight-fold lower. Other strains show that norfloxacin is affected to a greater extent than ofloxacin. Bazile and colleagues (1992) reported that in Gram-negative organisms, high molecular weight quinolones accumulate in the bacterial cell at a slower rate than those of a low molecular weight, and thus the MICs of norfloxacin and ofloxacin would be expected to be greater than those of ciprofloxacin.

The fluctuations in quinolone resistance in the paired clinical isolates, however, did not reflect the findings in the unpaired strains. The ratios of ciprofloxacin resistance to both ofloxacin and norfloxacin resistance either remained the same, or the norfloxacin/ciprofloxacin ratio was two-fold greater than the ofloxacin/ciprofloxacin ratio. These ratios were identical to those of the sensitive parent strains, and demonstrate that a similar resistance mechanism has occurred in all of the resistant isolates. The resistance of the paired clinical isolates was low, being 8mg/l or less to all of the quinolones investigated, suggesting that it was caused by permeability alterations (Hirai *et al* 1986a). The results indicated that resistance in the isolates 273 and 4269, which exhibit similar quinolone ratios to the paired isolates, was also by permeability mechanisms, and that the other unpaired resistant isolates had a second mutation, which acted in concert with the permeability mutations.

4.1.2 MICs of Other Antimicrobial Agents.

The observation that OMP alterations are partly responsible for quinolone resistance in *P.aeruginosa* was confirmed by the MIC experiments with other unrelated antimicrobial agents. *P.aeruginosa* was found to be intrinsically resistant to tetracycline, kanamycin, cefotaxime and carbenicillin. The levels of resistance to these drugs were different, however, ranging from 4-128 mg/l cefotaxime, 4-128 mg/l carbenicillin, 64->256 mg/l tetracycline and 2->128 mg/l kanamycin, which implied that at least two mechanisms of resistance to these drugs were present in the isolates studied. These mechanisms were probably permeability mutations coupled with plasmid-mediated resistance.

A study of the MICs of gentamicin, imipenem and ceftazidime in the clinical isolates demonstrated that quinolone resistance was linked with several different patterns of unrelated antimicrobial agent resistance. Three strains exhibited high-level quinolone resistance in conjunction with high-level gentamicin, imipenem and ceftazidime resistance, which indicated that drug resistance in these strains was caused by a membrane protein alteration. Strain C2 also exhibited high-levels of gentamicin, imipenem and ceftazidime resistance, but it was coupled with moderate quinolone resistance, and was thought to demonstrate the same resistance mechanism.

Several strains displayed quinolone resistance with resistance to two out of the three drugs, gentamicin, imipenem and ceftazidime; although in all cases resistance levels were variable. These strains exhibited quinolone resistances between 8 and 32 mg/l ciprofloxacin. Three strains were quinolone and ceftazidime resistant and one strain, 3, had high level quinolone resistance. Three strains also exhibited moderate gentamicin resistance, two of which had a ciprofloxacin MIC of 64mg/l. These results confirm that more than one resistance mechanism was present in these clinical isolates.

Quinolone and imipenem cross resistance has previously been reported by Rådberg and co-workers (1990) and Thomson and colleagues (1991) in quinolone-resistant clinical isolates of *P.aeruginosa*. Rådberg and co-workers, however, did not study resistance to gentamicin or ceftazidime and so could not link them to imipenem or quinolone resistance. In this study no strains were isolated which were solely quinolone- and imipenem-resistant. This suggests that the strains investigated by Rådberg and colleagues (1990) were also either gentamicin- or ceftazidime-resistant. Thomson and colleagues (1991) linked quinolone resistance in ciprofloxacin-resistant isolates of *P.aeruginosa* with imipenem, gentamicin and ceftazidime resistance, although resistance to these agents did not develop during the course of the study. Masuda and Ohya (1992) linked quinolone and imipenem resistance in laboratory mutants of *P.aeruginosa* and reported that most strains carried gentamicin and ceftazidime resistance. The imipenem/quinolone cross resistance in the report of Masuda and Ohya (1992) was linked to a permeability alteration in a 49kD protein (*OprM*).

Quinolone cross resistance with β-lactam antibiotics has also been reported (Rella and Haas 1982; Fukuda *et al* 1990) and was linked with alterations in drug permeability. Fukuda and colleagues (1990) discovered that β-lactam/quinolone cross resistance was initiated by a decreased expression of a 46kD protein and an increased expression of a 50kD protein. Fukuda and co-workers (1990) described similar alterations in MIC values to those observed in this study.

Quinolone resistance has been linked to hypersusceptibility to unrelated antimicrobial agents such as gentamicin, carbenicillin and β-lactam antibiotics, (reviewed by Yoshida *et al* 1990c). These reported hypersusceptibilities have been linked to decreases in expression of a 46kD protein and increases in expression of a 50kD protein (Fukuda *et al* 1990). Similar results were obtained in this study, with five moderately quinolone-resistant strains showing gentamicin sensitivities which were lower than those of the

drug-sensitive strains PAO1 and U423. Three strains also exhibited moderate quinolone resistance linked to ceftazidime sensitivity and two strains exhibited carbenicillin hypersusceptibility. One strain, C48, exhibited hypersusceptibility to all of the antimicrobial agents investigated, but was resistant to ciprofloxacin with an MIC of 32mg/l.

In the paired isolates from patients I and III, initial quinolone resistance was not linked with resistance to any unrelated antimicrobial agents. Only in later isolates of the 91-32 series from patient I did β-lactam resistance increase, and gentamicin and imipenem resistance increase by two-fold. This suggests that quinolone resistance can develop independently of resistance to unrelated antimicrobial agents, a finding which was reported by Hirai and colleagues (1986b) and was linked to DNA gyrase mutations. Quinolone resistance did not increase with the rise in β-lactam, gentamicin and imipenem resistance, which suggested that the resistance mechanism of these agents was separate to that of the quinolones. Mutations affecting resistance to these other agents were probably via pathways which did not involve quinolone uptake, or where quinolone uptake was minimal. In the paired isolates 90-62 and 90-67 quinolone resistance was linked to resistance to β-lactam antibiotics, although it was unclear whether these resistances developed independently of each other. As these two strains were isolated a month apart, it is possible that quinolone and β-lactam resistance could have developed independently and that this had not been observed. The β-lactam resistance probably developed in response to treatment with β-lactam antibiotics, and not with quinolone antibiotics, but the case histories of the patients were unavailable to confirm this.

The choice of media affected quinolone resistance, and this has been previously reported (Govan *et al* 1983; Bedard *et al* 1989). Govan and colleagues (1983) found that MICs were four-fold greater on PIA than on IST agar, and this was attributed to

the presence of the detergent, irgasan, in the media. This study obtained similar results. Bedard and co-workers (1989) reported that an increase in the magnesium ions in the media also contributes to higher MICs. The presence of excess Mg^{2+} is thought to prevent quinolones from chelating LPS-associated magnesium and so limits their entry into the bacterial cell (Chapman and Georgopapadakou 1988). PIA contains a much higher magnesium content than IST agar, which explained the discrepancies in MIC values.

The growth of *P.aeruginosa* strains on PIA also affected the colonial morphology of the clinical isolates. In 1983 Govan and colleagues reported that several strains of *P.aeruginosa* exhibited a mucoid colonial morphology on PIA but not upon any other media, which they determined PIA-dependent mucoidy. Several of the clinical isolates in this study exhibited mucoidy on PIA, which contributed to the higher quinolone MICs on this media compared to IST agar, as the viscous polymer, alginate, synthesised by the mucoid *P.aeruginosa* provides a very effective barrier to drug permeation into the bacterial cell. The conversion to mucoid colonies explained why the ciprofloxacin/ofloxacin resistance ratio in strain C2 increased on PIA and not on IST agar, and it also explained why the ciprofloxacin/ofloxacin resistance ratios between IST agar and PIA differ slightly in some strains.

Resistance to one 4-quinolone antibacterial leads to resistance to all 4-quinolone antibacterials, and this has been demonstrated by many authors (reviewed by Wolfson and Hooper 1989). Moniot-Ville and colleagues (1991) demonstrated, however, that resistance to the 4-quinolones was linked, but that 4-quinolone-resistant bacteria also exhibited susceptibility to nalidixic acid. This study did not investigate nalidixic acid resistance but the MIC results for the quinolones investigated do suggest that quinolones are affected in a variety of ways by different mutational changes.

4.2 THE STABILITY OF QUINOLONE RESISTANCE.

Investigations into the stability of quinolone resistance have generated conflicting reports. Daikos and colleagues (1988) reported that quinolone resistance in *P.aeruginosa* was abolished after repeated passage in drug-free medium. This finding was confirmed by Kaatz and Seo (1988) who reported that low level quinolone resistance linked to alterations in an OMP of 31-32kD was unstable. Rådberg and colleagues (1990) published a conflicting report which claimed that ciprofloxacin resistance in clinical isolates of *P.aeruginosa* from cystic fibrosis patients demonstrated little or no alteration in ciprofloxacin resistance after serial passage in Mueller-Hinton broth.

On initial observation, the results from thesis suggested that the findings of Rådberg and colleagues (1990) were correct, as in all strains ciprofloxacin resistance remained stable, or altered by two-fold after serial passage on drug-free PIA. This study did, however, isolate one strain, C48, which on one occasion reverted to ciprofloxacin sensitivity within five days of the start of the passage experiment. The sensitive revertant strain, C48s, exhibited an identical pyocin type to its parent strain. The strain C48 had demonstrated a high ciprofloxacin MIC of 32mg/l. Repetition of the experiment on two subsequent occasions revealed no reversion to sensitivity, indicating that ciprofloxacin resistance was stable in this strain. These results indicated, however, that on rare occasions the same strain may exhibit two different mutations to the 4-quinolones, one of which is stable in the absence of the selective agent, and one of which is unstable.

The isolation of these two different mutations might have been influenced by alterations in temperature, or the method used to investigate the stability of resistance. This study passaged a single colony from solid media onto a fresh plate, so it is possible that both ciprofloxacin-resistant and ciprofloxacin-sensitive colonies existed during the initial passage, but that only one phenotype was expressed on subsequent passages. This explained why reversion to ciprofloxacin sensitivity was only isolated on one occasion in the C48 strain. Rådberg and colleagues (1990) passaged their strains in Mueller-Hinton broth where a mixture of ciprofloxacin-resistant and ciprofloxacin-sensitive colonies would be inoculated into each fresh culture and MIC broth. The MIC broth would therefore always contain some ciprofloxacin-resistant strains, which would result in stable ciprofloxacin resistance being observed. Both Daikos and co-workers (1988) and Kaatz and Seo (1988) employed agar passage experiments which increased their ability to select ciprofloxacin-sensitive colonies. This theory could be investigated by performing viable counts on both ciprofloxacin-containing and drug-free agar plates.

4.3 ALTERATIONS IN OUTER MEMBRANE PROTEINS.

Alterations in OMPs which have been reported to be linked to quinolone resistance include proteins of 51kD (Robillard and Scarpa 1988), 31-32kD (Daikos *et al* 1988), 40kD (Chamberland *et al* 1989), 54kD (Hirai *et al* 1987), 50kD (Fukuda *et al* 1990), 47kD (Rohner *et al* 1992) and OMPG (Chamberland *et al* 1989). The MIC results in this study, indicated that strains cross-resistant to unrelated antimicrobial agents would probably contain membrane protein alterations. Strains exhibiting quinolone and imipenem resistance would be expected to express different OMP alterations from those exhibiting quinolone and gentamicin resistance, or quinolone and β-lactam resistance. It was expected that strains harbouring resistance to quinolones and the other three drugs would exhibit alterations in several OMPs.

4.3.1 OMP Alterations in the Unpaired Clinical Isolates.

This study found that it was difficult to identify OMP mutations as a majority of the clinical isolates were unpaired. It was attempted to overcome this by investigating the OMPs of several quinolone-sensitive isolates, and a reference strain PAO1, to decide whether a specific *P.aeruginosa* OMP profile predominated. It was unfortunate that the sensitive isolates exhibited different OMP profiles. One profile, however, did seem to dominate as it was observed in five of the sensitive strains; it contained OMPs at 51, 46, 43, 36, 26 and 22kD. This profile was then used as a standard with which to compare the rest of the clinical isolates.

The molecular masses of the OMPs of all the clinical isolates were calculated from PhastSystem gels. The BioRad Mini Protean® gels were chosen to be presented in this study because band differentiation, especially at the lower molecular masses, was clearer than that of the PhastSystem and because the gels were much larger in size. The proteins greater than 30kD, however, appeared to be cramped on the BioRad gels when compared to the PhastSystem gels, and so the molecular masses of the OMP bands were calculated from these gels.

In the unpaired isolates alterations in several OMPs were found; those of 51, 46, 43, 42, 38, 36, 32, 30 and 28kD proteins. The predominant alteration was a decrease in expression or total loss, of a 51kD protein. The production of an additional 51kD protein was found to be linked to quinolone resistance (Rella and Haas 1982; Robillard and Scarpa 1988), and isolates exhibited cross-resistance to chloramphenicol, tetracycline and carbenicillin. In this study the eight strains with a loss or decreased expression, of this protein were tetracycline-resistant, but four of the strains were carbenicillin-sensitive. Two strains, 4158 and 4161, exhibited low carbenicillin MICs linked with imipenem hypersusceptibility. The results therefore suggest that the 51kD protein may not be associated with quinolone/carbenicillin cross-resistance which

contradicts the report of Robillard and Scarpa (1988); however these workers investigated laboratory mutants of only one strain, PAO2.

Other investigators have implicated the presence of a 54kD protein in quinolone resistance (Hirai *et al* 1987; Legakis *et al* 1989). On observing the OMP profiles of the *P.aeruginosa* isolates investigated by Legakis and colleagues (1989), the protein at 54kD appears to be the identical protein to the protein in this study found to migrate to 51kD. Legakis and co-workers (1989) found that the increased production of this 54kD protein was linked to β-lactam and aminoglycoside cross resistance. This result contradicts the findings of this study, as although alterations to the 51kD protein were associated with increases in MIC of both aminoglycoside and β-lactam antibiotics, the 51kD protein expression was seen to decrease and not to increase. This might suggest that these two proteins were indeed different, although the OMP profiles seem to indicate otherwise.

Hashmi and Smith (1991) reported increased expression of a 52kD protein, which conferred high-level quinolone resistance, but no cross resistance to unrelated antimicrobial agents. Again, on studying the OMP profiles of Hashmi and Smith's isolates (1991), the 52kD protein appears to be identical to the 51kD protein in this thesis. Two strains, 4158 and 4161, were solely quinolone-resistant but displayed a decreased expression or loss, of a 51kD protein, which contradicted the report of Hashmi and Smith (1991) where the expression of the 52kD protein increased.

Decreased expression or the loss, of the 51kD protein was coupled with the production of a new 28kD protein and was linked with β-lactam resistance in four strains, suggesting that β-lactam resistance is caused by the synthesis of a new membrane-bound protein. The production of a new 28kD protein linked to quinolone resistance has been reported by Daikos and colleagues (1988) but their isolates were only quinolone-resistant and not resistant to any other antimicrobial agents. This indicated that the β-lactam resistance was caused by other mechanisms.

Alterations in a 51kD protein were also linked with the production of a new 38kD protein, although three other strains were observed to produce the new 38kD protein without visible alterations in the 51kD OMP. The production of a 38kD protein could not be linked with resistance to any other antimicrobial agent, and strains producing this extra protein showed different antibiograms. This suggests that the 38kD protein might be linked to quinolone resistance, but no other reports of increases in this protein have been documented.

Another frequent OMP alteration observed was the loss or decreased expression, of a 43kD protein. The loss of this protein and the production of a new 30kD protein was linked in two strains to an increase in gentamicin resistance. This correlates with the results observed by Daikos and colleagues (1988) who reported that quinolone resistance was caused by alterations in the expression of a 43kD and a 31-32kD protein.

Other protein alterations observed were those at 46 and 32kD. Decreased expression of protein D2, a 47kD protein, has been linked to quinolone/imipenem cross resistance in *P.aeruginosa* (Michéa-Hamzehpour *et al* 1991). In this study, however, the loss of a 46kD protein was not linked to any alterations in imipenem resistance. The production of a new 32kD protein in three strains was linked with quinolone and β-lactam

resistance but two of the strains also had alterations in a 51kD protein and produced two other new proteins at 38 and 28kD. Reports by Daikos and colleagues (1988) and Kaatz and Seo (1988) have implicated the loss or decreased expression of a 32kD protein in low level quinolone resistance, which again seems to contradict the findings of this study. It is clear, however, from considering the alterations in the unpaired clinical isolates in this study, and the reports of other investigators, that *P.aeruginosa* may exhibit several OMP alterations in conjunction with quinolone resistance.

It was also observed that all of the quinolone-resistant isolates contained a protein at 36kD. In isolates with ciprofloxacin resistance above 8mg/l the expression of this protein was significantly increased compared to its expression in sensitive strains. This suggested that this 36kD protein was involved in quinolone resistance.

4.3.2 OMP Alterations in Paired Clinical Isolates.

The paired isolates from patient II displayed no alterations in OMP profiles, which suggested that quinolone resistance was brought about by either a DNA gyrase mutation, usually associated with high level quinolone resistance, or the presence of an active efflux system. Neither of these suggestions were investigated further.

The paired isolates from patients I and III exhibited similar alterations in OMP profiles, and isolates from both patients acquired β-lactam resistance in conjunction with quinolone resistance. The resistant strains 90-67 and 91-33 displayed alterations in the production of a 46kD protein. The quinolone-resistant strain from patient III exhibited a decreased production of a 46kD protein, coupled with an increased expression of two proteins at 26kD and 22kD. In patient I, quinolone resistance was linked to the increased expression of a 46kD protein and the decrease of a 51kD protein. Development of ceftazidime resistance in patient I was linked to the production of a new 42kD protein. This protein was present in the membranes of both patient III's strains

and so β-lactam resistance in this strain probably developed independently of quinolone resistance.

4.3.3 OMP Alterations in Laboratory Mutants.

Results from both the unpaired and paired strains implicated three major proteins in quinolone resistance, those at 36kD, 46kD and 51kD. The results obtained from mutant strains confirmed these observations. The mutant strains from U423 exhibited an increased expression of a 36kD protein linked to an eight-fold increase in quinolone MIC. Two mutants also exhibited alterations in the expression of a 51kD protein, which might be linked to imipenem hypersusceptibility. Two other strains which demonstrated imipenem hypersusceptibility, however, did not display any visible alterations in the expression of the 51kD protein. The strains with imipenem hypersusceptibility were all green pigmented, and demonstrated similar pyocin types, so imipenem sensitivity might be linked to pyocin production and not to quinolone resistance.

Mutant isolates were also obtained from 91-32 and 91-33; the mutational frequencies observed of 2.1 in 10^6 and 5.4 in 10^6 were within the range reported by Wolfson and Hooper (1989) for *P.aeruginosa* isolates, although they were more frequent than the values quoted by Robillard and Scarpa (1988) of 1.5 in 10^7. Mutant bacteria from the strain 91-32 displayed variable quinolone resistances, unlike the mutant strains from U423, suggesting that more than one mutational step had occurred in strain 91-32 to produce these isolates.

The mutant laboratory strains from 91-32 exhibited similar OMP profiles to the ciprofloxacin-resistant clinical isolates obtained from this strain. The mutant isolates 91-32-1, 91-32-3 and 91-32-6 exhibited identical profiles to the clinical isolates 91-44 and 91-76. Their MICs , however, were very different. The three mutant strains exhibited

gentamicin resistance, whereas the clinical isolates were slightly gentamicin-resistant, and none of the laboratory mutant strains had the high levels of ceftazidime resistance demonstrated by their clinical counterparts. The mutant strains were also more sensitive to cefotaxime than the clinical isolates. These results were reflected by strains 91-32-2 and 91-32-4 which displayed identical OMPs to the clinical isolates 91-40 and 91-41. The mutant strains, however, possessed lower cefotaxime resistances and were ceftazidime-sensitive, unlike their clinical counterparts. This again suggested that the β-lactam resistance observed was linked to the patient's drug therapy and not to quinolone resistance.

MIC evaluations on the laboratory mutants of 91-32 showed that strains exhibiting high ofloxacin and ciprofloxacin MICs were imipenem-sensitive and, in some cases, gentamicin hypersusceptible. Several other strains with similar ofloxacin MICs exhibited gentamicin resistance and so sensitivity to gentamicin was disregarded. Hypersusceptibility to imipenem, however, had also been observed in the ciprofloxacin-resistant U423 laboratory mutants. This imipenem hypersusceptibility and quinolone cross resistance appeared to be linked to the production of a 36kD protein.

In order to investigate whether imipenem hypersusceptibility was linked to the increased production of a 36kD protein and if it was co-linked with quinolone resistance, the ciprofloxacin-resistant clinical isolate, 91-33, was selected further for mutants exhibiting high ofloxacin resistance. If imipenem hypersusceptibility was linked to quinolone resistance then the imipenem resistance of the mutant strains should alter. Ten mutant isolates, exhibiting various quinolone MICs, were studied and no alterations in imipenem resistances were observed. These results indicate that imipenem and ciprofloxacin resistance are not linked and, as ciprofloxacin resistance seems to be linked to alterations in 36kD and 51kD proteins, imipenem hypersusceptibility can not

be linked to alterations in either of these proteins. Unlike Rådberg and colleagues (1990), alterations in imipenem resistance could not be linked to either quinolone resistance or alterations in OMPs. It is possible that the imipenem resistance reported by Rådberg and colleagues (1990) could have resulted from the patients' treatment rather than in concert with quinolone resistance.

Experiments to confirm that either the 36kD or the 51kD proteins were responsible for quinolone resistance in the clinical strains, by reverting the resistant clinical strains to sensitivity, were unsuccessful. The experiments demonstrated that quinolone resistance was stable in these strains, and not surprisingly, no alterations in OMPs were found. These results from the clinical strains confirm the results previously observed in the unpaired isolates and demonstrate that, for the purpose of quinolone resistance, alterations in OMPs appear to be permanent. The reversion of C48 to sensitivity suggests that two types of OMP mutations may occur, but that detection of the sensitive strains is difficult.

4.3.4 OMP Alterations in the Presence of EDTA.

To clarify which of the quinolone-resistant strains carried OMP alterations, the MICs of quinolones for several strains were monitored in the presence of increasing concentrations of EDTA. Previous work with trimethoprim (Amyes and Smith 1977) and quinolones (Sato *et al* 1986) has demonstrated that permeability mutations may be detected by performing MICs in the presence and absence of EDTA. Disruption of the bacterial outer membrane with EDTA has been reported to facilitate quinolone uptake by opening the hydrophobic pathway to them (Chapman and Georgopapadakou 1988). It would therefore be expected that strains harbouring OMP mutations would be affected by the presence of EDTA and that in these cases the quinolone MIC would be seen to decrease.

EDTA concentrations above 8mM were found to inhibit bacterial growth, which is not surprising, as disruption of the cell membrane would allow osmotic changes to occur, and the chelation of divalent cations in the bacterial cell by excessive EDTA molecules would prevent many essential cell processes from taking place. EDTA had no effect upon the sensitive bacteria, until the 8mM EDTA concentration was reached. The resistant strains, however, exhibited varying reactions at different EDTA concentrations. All of the strains demonstrated significant decreases in quinolone MIC when subjected to concentrations of 8mM EDTA; although 11 out of the 24 resistant isolates investigated managed to grow at these concentrations. These results suggest that all of the strains possessed OMP alterations, but that several strains also possessed other resistance mechanisms.

Most strains displayed gradual decreases in quinolone MIC with increasing EDTA concentrations, suggesting that in these strains OMP mutations were the primary resistance mechanism. The quinolone-resistant mutant of the paired strain 91-32, 91-33, exhibited a four-fold decrease in quinolone MIC at 2mM EDTA concentrations, which was linked to the decreased expression of a 51kD protein and increased expression of a 46kD protein. Four strains also exhibited similar alterations in quinolone MIC, which were also linked to the altered expression of a 51kD protein. Strains 3, 4124, 4161 and 4495 showed different alterations in quinolone resistance in the presence of EDTA, suggesting that they exhibited other quinolone resistance mechanisms, and possibly different OMP alterations.

At concentrations of 4mM EDTA more than 50% of the resistant isolates exhibited quinolone sensitivity. The remaining isolates exhibited MICs of 4mg/l ciprofloxacin or greater, indicating that they possessed quinolone resistance mutations other than permeability mutations. These mutations were thought to be gyrase mutations, and not efflux mutations, as the disruption of the outer membrane would affect the mechanism of action of an efflux system.

The EDTA results confirmed that all of the resistant strains possessed OMP mutations. Alterations in quinolone MICs within the resistant bacteria were not linked to alterations in specific membrane proteins, although strains exhibiting the loss or decreased expression of a 51kD protein did show similar falls in quinolone MICs. The alterations in quinolone MICs in the presence of EDTA were similar to those reported by Sato and co-workers (1986). The fact that specific OMPs could not be linked to changes in EDTA concentrations seems to confirm that in *P.aeruginosa* several OMP mutations, acting separately or in conjunction with each other, are responsible for quinolone resistance. The presence of other resistance mechanisms in these strains, such as gyrase mutations, also contributed to the variable quinolone MICs in the presence of different amounts of EDTA. Strains carrying mutant DNA gyrases would be expected to exhibit higher initial quinolone MICs in the presence of EDTA, as there would be insufficient EDTA in the medium to affect the inner cell contents. As EDTA concentrations increase, Mg^{2+} ions in the cell would be chelated, and gyrase-mediated quinolone resistance would be inhibited. This caused the sudden decrease in quinolone resistance seen in eight strains.

The study of MIC alterations in the presence of EDTA implicated four different alterations in membrane proteins, as four patterns of quinolone resistance emerged; an alteration in ciprofloxacin/ofloxacin resistance or ciprofloxacin/norfloxacin resistance ratios being indicative of a specific permeability change. The comparison of quinolone

ratios with permeability changes showed no correlation. The results suggested that different quinolones may be affected to varying extents by permeability alterations; the extent of the effect on quinolone permeation into the cell depending upon the permeation route of the quinolone investigated. Reports by Hirai and colleagues (1986a) suggest that the more hydrophobic quinolones, for example nalidixic acid and oxolinic acid, would be less affected by permeability changes than the hydrophilic quinolones, ciprofloxacin and norfloxacin, as they are able to diffuse directly through the outer membrane.

Permeability alterations also affect quinolone accumulation in the cell, and through this the MIC, as they enable efflux mechanisms to play a significant role; as less quinolone molecules penetrate the cell, an efflux mechanism would exert more effect and quinolone MICs would be expected to become higher. The disruption of the cell membrane with EDTA influences the actions of any efflux mechanism, allowing quinolones to diffuse in and out of the bacterial cell. This explains why the norfloxacin MICs dropped considerably in the presence of EDTA, as norfloxacin has been shown to be excreted from *E.coli* cells (Cohen *et al* 1988) and it is likely that a similar mechanism may exist in *P.aeruginosa.*

The proteins of 51kD, 46kD, 43kD, 36kD, 32kD and 28kD were observed to be non-covalently associated with peptidoglycan, as they remained with the peptidoglycan fraction after solubilisation with SDS. They were also released from the peptidoglycan fraction after heating in SDS which suggested that they might function as porins. Hirai and colleagues (1986b) reported that the major entry route for quinolones into the bacterial cell was through porin pathways, and that alterations in porin proteins increased quinolone MICs. The results of this study confirm this report, as alterations in suspected porin proteins brought about increases in quinolone MICs.

4.4 ALTERATIONS IN DNA GYRASE.

Previous work in both *E.coli* (Hirai *et al* 1986b) and *P.aeruginosa* (Hirai *et al* 1987; Inoue *et al* 1987) has demonstrated that mutations in DNA gyrase cause high-level quinolone resistance. These studies on *P.aeruginosa* only investigated the presence of gyrase mutations in two ways, either a) by separating the α and β subunits of DNA gyrase and recombining them with wild-type, sensitive, α and β subunits or b) by using plasmid probes expressing wild-type, and thus quinolone-sensitive, gyrase genes.

The first method of separating gyrase subunits, employed by Robillard and Scarpa (1988) and Inoue and colleagues (1987), although demonstrating the presence of DNA gyrase mutations, is very inaccurate. On separating and reconstituting DNA gyrase, investigators pick a ratio of α to β subunit which supercoils the largest amount of pBR322, and this is not necessarily the ratio of α to β subunits present in the bacterial cell. It is also difficult to ensure that all of the gyrase subunits have reconstituted to form a single enzyme, and so the results, although valid are inaccurate and can not be compared with other bacteria (Dr K. Sato personal communication).

The second technique used plasmid or cosmid vectors carrying sensitive gyrase genes, which caused bacteria to revert to quinolone sensitivity. This technique has recently been questioned by Soussy and co-workers (1993) whose experiments indicated that this method is unreliable, produces false negative results, and overestimates the level of *gyrA* resistance in many cases.

This study investigated *gyrA* mutations in *P.aeruginosa* directly, by isolating the whole DNA gyrase enzyme and determining its ability to supercoil relaxed pBR322 in the presence of several antimicrobial agents, and by amplifying the active site of the *gyrA* gene by PCR methods. Two indirect methods were also investigated; the first involved

the amplification of the *gyrA* gene by PCR methods and the restriction of the reported active site with *SstII*, and the second involved probing for *gyrA* mutants with a plasmid containing the sensitive *E.coli* wild type *gyrA* gene.

4.4.1 Determination of DNA Gyrase Activity.

This study, unlike previous studies on gyrase resistance in *P.aeruginosa* (Inoue *et al* 1987; Robillard and Scarpa 1988) studied gyrase mutations in a large number of clinical isolates obtained from a wide variety of sources. The effect of three quinolones, ciprofloxacin, ofloxacin and norfloxacin, and one courmarin, novobiocin, upon the ability of the isolated gyrase to supercoil relaxed pBR322 was investigated. The quinolones were chosen as they are available clinically, and demonstrated the presence of *gyrA* mutations, and novobiocin was selected as it indicated the presence of *gyrB* mutations.

The IC_{50} of ciprofloxacin, the most widely prescribed 4-quinolone, fell into three broad categories. The majority of isolates belonged to a group with low IC_{50} values which were at least 100-fold greater than the ciprofloxacin IC_{50}s of the quinolone-sensitive reference strain, PAO1. The remaining isolates were split equally between two groups, those with moderate ciprofloxacin IC_{50}s and those with very high ciprofloxacin IC_{50}s. The initial hypothesis of this study was that strains in these IC_{50} groups might exhibit similar, or identical gyrase mutations. All of the clinical isolates investigated exhibited gyrase mutations.

Further interpretation of the results demonstrated that the ofloxacin and norfloxacin IC_{50}s did not mirror the ciprofloxacin results, and that strains with low ciprofloxacin IC_{50}s could exhibit high ofloxacin and norfloxacin IC_{50}s. This questioned the accuracy of measuring IC_{50}s. It is impossible to compare directly IC_{50} values between strains, as the method employed does not quantify the amount of DNA gyrase present in each

sample. Also, the DNA gyrases isolated have not been completely purified, and so the amount of enzyme lost in each strain as a result of the purification process might be unequal. Strains exhibiting high IC_{50} values might contain larger amounts of gyrase than those exhibiting low IC_{50} values, or they might contain gyrase in a more active state. The activity of DNA gyrase is hard to establish, as cells must be caught at the correct growth phase to isolate large amounts of active gyrase. This study found, for example, that three times as much cell weight was needed to isolate gyrase from strain 3, compared with strain C32, although both strains exhibited similar IC_{50} values.

Bazile and colleagues (1992) have evaluated gyrase activity by another parameter, the minimal effective dose (MED) which is the minimum amount of drug required to cause any inhibition of DNA gyrase activity. They reported that MED values are more accurate than IC_{50} values and that they are easier to evaluate by gel electrophoresis. Bazile and co-workers (1992) were also able to compare MED values between strains after applying a correction factor for the bacterial accumulation of quinolones. This study chose to investigate IC_{50} values, as these were the classical values expressed in all previous reports.

Initial attempts to quantify the activities of DNA gyrase from 4131 and 4133 gave very low IC_{50} results, which on re-isolating the DNA gyrase became 5- to 10-fold higher (results not shown). These results suggested that the discrepancies between the IC_{50} groups was caused by differences in DNA gyrase-specific activities, rather than in the actual gyrase mutations themselves.

The discrepancies in IC_{50} values between low and intermediate IC_{50} groups, and the intermediate and high groups, can also be explained as not all IC_{50} assays were performed immediately after the isolation of DNA gyrase. Some DNA gyrases were stored for 4-5 weeks before IC_{50} assays were undertaken. This accounts for slightly

lower IC_{50} values in some cases, as DNA gyrase activity has been shown to decline with storage (Sato *et al* 1986). Reports by Gellert and colleagues (1976) and Inoue and co-workers (1987), however, indicate that IC_{50} values do not decline noticeably during one month's storage of gyrase, so any decline in IC_{50} value because of storage would be unlikely to explain the 10-fold changes in IC_{50} values between the low and high IC_{50} groups.

The validity of the IC_{50} results were investigated further by sequentially diluting the DNA gyrases of two strains and determining the new ciprofloxacin IC_{50} values. Both strains demonstrated that dilution of the DNA gyrase reduced the IC_{50} value by the same factor; a 50% dilution of the DNA gyrase resulted in a 50% reduction of the IC_{50} value. This reduction in values, however, was insufficient to explain the ten-fold differences in gyrase activities between DNA gyrases in the low and high IC_{50} groups, indicating that these groups displayed different gyrase mutations.

The dilution of the DNA gyrase produced another interesting result as, unlike most enzymatic assays where decreasing the enzyme concentration has little or no effect upon the final result, decreasing the gyrase concentration in this assay had noticeable effects on the IC_{50} results. This suggested that the quinolones were actually binding to a DNA/DNA gyrase complex and not to the DNA or to the DNA gyrase themselves, and confirms the findings of Shen and colleagues (1989) and Willmott and Maxwell (1993). The sequential dilution of DNA gyrases demonstrated that the ratio of supercoiled to relaxed pBR322 did not alter significantly (results not shown) until very small amounts of gyrase were present. This again confirms that quinolones bind preferentially to a DNA/DNA gyrase complex and not to the single constituents themselves.

The difference in ratio of IC_{50} values between quinolones was also investigated, as it has been proposed that the differences in IC_{50} ratios between quinolones, and not the differences in IC_{50} values of a single quinolone, indicate the presence of a specific gyrase mutation (Dr K. Sato, personal communication). This study found that no distinct groups could be determined on the basis of quinolone resistance ratios, although several strains did exhibit the same basic trend. Dr Sato, however, based his hypothesis on the IC_{50} ratios of at least eight quinolone antimicrobial agents, and by only using three quinolones in this study it is likely that insufficient data were obtained to group the clinical isolates in this way.

4.4.2 Relationship Between IC_{50} and MIC.

The comparison of IC_{50} and MIC values corroborated the findings of other investigators, (Inoue *et al* 1987), that MIC values are generally lower than IC_{50} values. This is because quinolones are thought to accumulate in the interior of the bacterial cell (Yoshida *et al* 1990b), therefore subjecting the DNA gyrase to much higher quinolone concentrations than those in the external medium. The relationship between MIC and IC_{50} values has also been investigated. Jin and Piddock (1993) reported correlations between MIC and IC_{50} values in ciprofloxacin-resistant laboratory mutants of *Streptococcus pneumoniae,* and Piddock and Zhu (1991) reported correlations between MIC and IC_{50} values for sparfloxacin and ciprofloxacin in several bacterial strains. These reports contradict the findings of this study, where no correlation was observed between MICs and IC_{50} values for any of the quinolones studied. Jin and Piddock (1993), however, only considered the MIC and IC_{50} value relationship in five laboratory mutants of *S.pneumoniae*, whilst Piddock and Zhu (1991) only investigated one strain of *P.aeruginosa.* This study contained 23 strains, so the investigation here into the relationship between IC_{50} and MIC is likely to be more accurate.

Direct comparison between MIC and IC_{50} values assumes that bacterial cell growth is inhibited by inhibiting the activity of DNA gyrase by 50%, but this assumption has not been proven. Bazile and colleagues (1992) compared MICs in *E.coli* isolates with the MED, and found that for most of the quinolones studied a positive correlation was obtained. The MED is a more accurate parameter with which to compare MIC values, but Bazile and colleagues (1992) observed that accurate results were only obtained when a drug accumulation correction factor was applied to the MED. Bazile and co-workers (1992) also found that the MED was higher than the MIC, which mirrored the observations for IC_{50} values in this study.

The relationships between the quinolone IC_{50}s indicated that various quinolone antimicrobial agents are affected to different extents by different gyrase mutations. This has been linked to the binding affinity of the drug for the DNA/DNA gyrase complex. Yoshida and co-workers (1993) have reported that enoxacin binds at two different frequencies to the DNA/DNA gyrase complex, and that mutations in the gyrase enable some drugs to bind more easily than others, thus affecting IC_{50} values. The model of quinolone binding to the DNA/DNA gyrase complex proposed by Shen and colleagues, (1989), also suggests that alterations in the DNA gyrase configuration may have several effects upon different quinolone structures.

4.4.3 Determination of DNA Gyrase Activity in Laboratory Mutants.

Mutant isolates were selected from strains C32 and 4149 until they had MIC values of 32mg/l ciprofloxacin, and the OMP profiles and DNA gyrase activities of these mutant strains were then investigated. Neither strain had demonstrated visible OMP alterations previously, but on investigating the OMPs after the mutation experiments, 4149^R exhibited an extra 38kD protein. The production of an extra 38kD protein had been previously observed in six of the clinical isolates, and was linked to an increase in

quinolone resistance. No alteration in OMP profile of the ciprofloxacin-resistant mutant of C32 was observed.

The mutant of 4149, 4149^R, also exhibited a three-fold increase in quinolone IC_{50} compared to its parent strain, which suggested the presence of a low level resistance gyrase mutation, and the novobiocin IC_{50} value was observed to decrease three-fold. The results from the strain $C32^R$ were inconclusive, as the drug concentrations used in the initial assay were too low to give a clear IC_{50} value. There was not, however, a three-fold increase in IC_{50} values as seen in 4149^R. The novobiocin IC_{50} value of $C32^R$, like that of 4149^R, did fall two-fold. The reason for this fall in novobiocin IC_{50} is unclear, but alterations in the *gyrA* subunit might confer a conformational change in that subunit which alters the relationship between the *gyrA* and *gyrB* subunits, and thus facilitates novobiocin binding to the β subunit. This result confirms the theory of Yoshida and colleagues (1993) that alterations in *gyrA* may affect the *gyrB* subunit.

GyrB mutations were also present in some unpaired clinical isolates in this study. It was observed that two strains, C32 and 3, both in the high IC_{50} group, exhibited novobiocin IC_{50} values which were seven-fold greater than those of the sensitive strain PAO1, and five-fold greater than those of the other clinical isolates. This indicated that these two strains contained *gyrB* mutations. Three strains 4149, 4374 and 4352 exhibited novobiocin IC_{50} values three-fold greater than PAO1, and might also harbour *gyrB* mutations. These findings corroborate reports by other investigators (Yoshida *et al* 1990b) that *gyrB* mutations are much less frequent than *gyrA* mutations, and thus would be expected to have a lesser effect upon bacterial resistance. Strains exhibiting suspected *gyrB* mutations were not investigated further as there was insufficient time.

4.4.4 Relationship Between OMP Alterations and DNA Gyrase Mutations.

Quinolone-resistant clinical isolates of *P.aeruginosa* which exhibited permeability alterations might be expected to possess gyrase mutations conferring low level resistance, that is low IC_{50} levels. This is because OMP alterations would reduce the uptake of the quinolone antimicrobial agents into the cell, and in the case of norfloxacin allow any efflux mechanisms to exert a greater effect on overall quinolone accumulation in the cell. The gyrases of bacteria possessing OMP mutations would therefore be subjected to lower levels of quinolones in the cell, and are more likely to develop gyrase mutations conferring low-level quinolone resistance. Conversely, those resistant bacteria with no visible membrane protein alterations should contain DNA gyrases with high levels of quinolone resistance, as there is no restraint upon quinolone accumulation in the cell.

The results contradict this assumption. High IC_{50} values were observed in three strains, C1, C32 and C49, with no visible OMP mutations, however, the remaining two strains with high ciprofloxacin IC_{50} values, 3 and 4374, did exhibit OMP alterations; strain 3 lost a 51kD protein and gained a 28kD protein, whereas strain 4374 gained a 38kD protein and exhibited decreased expression of a 51kD protein. Strain 3 did however have an elevated MIC compared to the other strains in this group, and the OMP alteration might have contributed to this four-fold increase in MIC, but did not otherwise affect the gyrase mutation.

Three strains C2, 271 and 4149, exhibited low IC_{50} values with no visible OMP changes, whilst still exhibiting ciprofloxacin MICs of 32, 8 and 8 mg/l, respectively. These strains should have exhibited moderate to high IC_{50} levels as no other quinolone resistance mechanisms were detected. The absence of paired strains, however, does not allow us to assume that these strains exhibited no OMP alterations. Indeed, the EDTA

results suggested that all of the clinical strains displayed OMP mutations, and so the techniques employed in this study are obviously not adequate to detect minor changes in protein expression. Uptake studies on these bacteria might have demonstrated the presence of OMP alterations or efflux mechanisms, but again, without the original parent strain as a reference the results would be inconclusive.

Eight strains exhibited low IC_{50} values in conjunction with visible OMP mutations, as expected, and the remaining strains with moderate IC_{50} values all demonstrated OMP alterations. The level of IC_{50} resistance, however, could not be linked to alterations in specific OMPS, but again this may be because the strains were unpaired and so slight alterations in OMPs might not have been detected.

4.4.5 Direct Sequencing of the *P.aeruginosa GyrA* gene.

GyrA gene mutations were investigated by amplifying a region of 300 base pairs, incorporating the 'quinolone resistance-determining region' by PCR methods. This study attempted to sequence the 300 base pair product of the PCR reaction directly using a technique with DMSO (Winship 1989). This technique was found to produce multiple bands at each nucleotide, producing an unreadable sequence. To combat this the annealing times of the primers were lowered, as reproduction of bands is indicative of primer mismatching and reannealing. Attempts to modify this technique were unsuccessful, and it was hoped to use single-strand sequencing techniques using biotin labelled probes instead, but there was insufficient time in this study.

It would be expected, from studying the *P.aeruginosa* amino acid sequence of the *gyrA* gene, that mutations would occur from threonine 83 to isoleucine to cause high level quinolone resistance. This alteration has been documented for *C.jejuni* (Wang *et al* 1993), which is the only other bacteria possessing a threonine residue at the active site. As the *gyrA* sequence of amino acids in *P.aeruginosa* is almost identical to that of

E.coli, mutations causing amino acid alterations in *E.coli* would also be expected to occur in *P.aeruginosa*. This study was unable to sequence double-stranded DNA directly by DMSO techniques, so it was impossible to confirm the *gyrA* sequence of *P.aeruginosa*, donated by L.E. Bryan. The PCR reactions using specific primers from this sequence were successful, however, which suggests that the sequence was correct.

4.4.6 Indirect Analysis of the *GyrA* gene.

The presence of threonine at the active site of *P.aeruginosa* was also verified using indirect methods. PCR products were restricted with the enzyme *SstII* which cleaves the *P.aeruginosa* quinolone-sensitive *gyrA* gene between amino acids 83 and 84, and recognises a sequence of six nucleotides within this region (refer to Fig 3.20 p132). Amino acid alterations from threonine to isoleucine will change the second base in this codon from cytosine to thymine, and mutations of alanine 84 to valine will again alter the second base, cytosine, to thymine. Both these changes would then prevent restriction by the *SstII* enzyme and thus indicate *gyrA* mutations at this site.

The results demonstrated that half of the bacterial strains investigated carried alterations at either amino acid 83 or amino acid 84. Without sequencing the PCR product this study was unable to determine which amino acid had been altered. From previous investigations on *E.coli, S.aureus* and *K.pneumoniae* it is likely that the majority of alterations would occur from Thr83 to isoleucine, which causes high level resistance in *C.jejuni.*

The analysis of the eight strains with gyrase mutations around amino acids 83 and 84 demonstrated that only one strain, C1, belonged to the high IC_{50} group. Of the remaining strains two, 4124 and 4161, were in the intermediate IC_{50} group and five strains demonstrated low IC_{50} levels of ciprofloxacin. Of the nine strains which were uncleaved, and therefore did not carry mutations at amino acids 83 and 84, two strains,

C32 and 4374, exhibited high IC_{50} values of ciprofloxacin. Again, of the remaining strains two, 4133 and 4375, belonged to the intermediate group and five belonged to the low IC_{50} group.

These results are unexpected, as it was thought that strains carrying amino acid alterations at position 83 would exhibit high ciprofloxacin IC_{50} levels. There are several explanations as to why these results have occurred. The first is that the IC_{50} results are unreliable, as the levels of DNA gyrase in the assay were not quantified. The dilution of DNA gyrase lowers IC_{50} values, and so strains in the low IC_{50} group harbouring gyrase mutations might exhibit medium and high levels of quinolone resistance, but these have remained undetected.

An alternative suggestion is that strains with high IC_{50} levels could harbour several amino acid alterations, and that the threonine to isoleucine and the alanine 84 to proline amino acid alterations only confer medium level resistance, which in concert with another amino acid alteration cause high IC_{50} values. Strains C32 and 4374 which were not cleaved by *SstII* would be expected to carry amino acid alterations in other parts of the quinolone-determining region. As the amino acid sequence of the *gyrA* gene is highly conserved, possible mutations could occur in amino acid 87 from aspartic acid to asparagine, as this has been documented to cause medium and high-level quinolone resistance in *E.coli* (Yoshida *et al* 1990a). This study was unable to obtain restriction enzymes which recognised mutations in nucleotide sequences around this site, or at other sites known to harbour base changes in *E.coli*, and so this possibility could not be investigated further.

Strain C32 was also thought to harbour mutations in the *gyrB* gene, and this coupled with low or medium resistance mutations in the *gyrA* gene might have led to a high IC_{50} value. Strains 4374, 4375 and 4149 also demonstrated novobiocin IC_{50} levels

which were three-fold greater than that of the reference strain PAO1, and these too might harbour *gyrB* mutations which, coupled with low level *gyrA* resistance could contribute to the medium and high levels of ciprofloxacin IC_{50}s observed.

The *SstII* cleaved strains with low IC_{50} levels would be expected to carry amino acid mutations at threonine 83 which conferred low level quinolone resistance, although no mutations in *C.jejuni* conferring low level quinolone resistance have been documented. An alternative suggestion is that these strains carry alterations in amino acid 84 which gives medium level resistance to quinolones in *E.coli.*

4.4.7 Analysis of the *GyrA* gene with pNJR3-2.

In order to determine whether the clinical isolates carried *gyrA* mutations an attempt was made to introduce a plasmid carrying the wild-type *E.coli gyrA* gene into *P.aeruginosa*. Previous work by Robillard (1990) and Piddock and colleagues (1992) had demonstrated that the introduction of a sensitive *gyrA* gene into *P.aeruginosa* resulted in the reduction of quinolone MIC, if the strain also carried OMP or efflux mutations, or complete reversion to quinolone sensitivity, if the strain carried only DNA gyrase mutations. Both groups have been successful in introducing the probe pNJR3-2 into *P.aeruginosa* by conjugation and transformation methods.

Attempts to introduce the probe into *P.aeruginosa* were unsuccessful, as the clinical isolates of *P.aeruginosa* in this study exhibited high intrinsic resistance to the selection markers on the probe, tetracycline and kanamycin. Alternative conjugation experiments, with different methods from those of Robillard (1990), were also unsuccessful, again because of the selection techniques. Experiments with EDTA demonstrated that most of the clinical strains retained their tetracycline resistance in the presence of EDTA, suggesting that, in these isolates, tetracycline resistance was carried by plasmid determinants.

Attempts to introduce pNJR3-2 into PAO1, which demonstrated lower tetracycline MICs than the majority of the clinical isolates in this study, were also unsuccessful. Investigations into the mobility of the plasmid demonstrated that the plasmid could mobilise in the same strain, which suggested that the transfer problem was likely to be caused by strain differences between *P.aeruginosa* and *E.coli*. To overcome this it was decided to introduce pNJR3-2 into *P.aeruginosa* by electroporation methods previously reported by Diver and colleagues (1990a).

Again, attempts to introduce pNJR3-2 into *P.aeruginosa* by electroporation methods were unsuccessful, because of impurities in both the plasmid and bacterial preparations, which lead to excessive arcing when the resistivity of the Electrocell Manipulator was increased. Arcing is caused by the presence of excessive salts, and it is likely that the plasmid extraction method of Takahashi and Nagano (1984) did not provide plasmid DNA with sufficiently low salt concentrations to prevent arcing from occurring.

Reports by Heisig and Wiedemann (1991) have suggested that pNJR3-2 is too large to successfully introduce into *P.aeruginosa,* as they obtained very low transfer frequencies in their experiments. Heisig and Wiedemann (1991) have successfully transformed the *E.coli gyrA* gene into *P.aeruginosa* with a smaller plasmid, pBP515, and reported that transformation was stable and occurred at a greater frequency than that of pNJR3-2.

Recent reports by Soussy and colleagues(1993), however, have questioned the overall validity of performing these experiments. Soussy and co-workers (1993) reported that introducing sensitive *gyrA* genes by transformation techniques produced many false negative results, caused by plasmid instability. This suggested that pNJR3-2 was probably unstable in *P.aeruginosa* and explained why no transconjugants reverted to quinolone sensitivity.

4.5 CONCLUDING OBSERVATIONS.

To summarise, quinolone resistance in *P.aeruginosa* occurs by several mechanisms, either by permeability changes, gyrase mutations, efflux mechanisms, or a combination of the three. This study indicated that permeability and gyrase alterations are pleiotropic in nature, and that all of the unpaired clinical isolates investigated contain both types of mutations. This begs the question of which resistance develops first, the impermeability mutation or the gyrase mutation? Work with other antimicrobial agents suggests that permeability alterations occur first, which reduce the effect of the antimicrobial agent on the bacteria, allowing it to develop other resistance mechanisms. The EDTA results demonstrated that each strain investigated carried membrane mutations, which suggests that OMP mutations occur before gyrase mutations. This study, however, could not resolve the question completely, as no strains were isolated which exhibited a sole quinolone resistance mechanism.

The OMP mutations observed were in a variety of proteins, but all of the ciprofloxacin-resistant isolates exhibited an increased expression of a 36kD protein. Many isolates also exhibited a decrease in the expression of a 51kD protein, which suggested that in *P.aeruginosa* alterations in these two proteins were responsible for the majority of quinolone resistance. Alterations in a 46kD protein, in the paired clinical isolates, were also shown to be important in quinolone resistance. The DNA gyrases of the paired strains were not investigated because these strains exhibited low level quinolone resistance and were still classified as quinolone sensitive by the BSAC guide-lines (1991). It would be interesting to determine whether these isolates contained only permeability mutations, or whether gyrase mutations had also developed.

This study also demonstrated that the measurement of IC_{50} values might not be an accurate method for determining the extent of DNA gyrase mutations, and that PCR and direct sequencing methods, as employed in *E.coli* and other bacteria are superior techniques. This study, like Soussy and colleagues (1993) also questions the validity of employing conjugation, transformation and electroporation techniques in the determination of DNA gyrase mutations.

REFERENCES.

Amyes,S.G.B. and Gould,I.M. (1984). Trimethoprim resistance plasmids in faecal bacteria. *Annales de Microbiologie de l'Institut Pasteur* **135B**: 177-186.

Amyes,S.G.B. and Smith,J.T. (1974). R-factor trimethoprim resistance mechanism: An insusceptible target site. *Biochem. Biophys. Res. Commun.* **58**: 412-418.

Amyes,S.G.B. and Smith,J.T. (1976). The purification and properties of the trimethoprim-resistant dihydrofolate reductase mediated by the R-factor, R388. *Eur. J. Biochem.* **61**: 597-603.

Amyes,S.G.B. and Smith,J.T. (1977). Trimethoprim resistance controlled by a combination of plasmid and chromosomal genes. *Genet. Res.* **29**: 35-45.

Amyes,S.G.B. and Towner,K.J. (1990). Trimethoprim resistance; epidemiology and molecular aspects. *J. Med. Microbiol.* **31**: 1-19.

Amyes,S.G.B., Payne,D.J. and DuBois,S.K. (1992). Plasmid-mediated β-lactamases responsible for penicillin and cephalosporin resistance. **In:** *Antibiotic Resistance in Bacteria.* (Amyes,S.G.B. and Gemmell,C.G. eds). *J. Med. Microbiol.* **36**: 4-9.

Aoyama,H., Fujimaki,K., Sato,K., Fujii,T., Inoue,M., Hirai,K. and Mitsuhashi,S. (1988a). Clinical isolate of *Citrobacter freundii* highly resistant to new quinolones. *Anti. Agents and Chemother.* **32**: 922-924.

Aoyama,H., Sato,K., Fujii,T., Fujimaki,K., Inoue,M. and Mitsuhashi,S. (1988b). Purification of *Citrobacter freundii* DNA gyrase and inhibition by quinolones. *Anti. Agents and Chemother.* **32**: 104-109.

Barrett,J.F., Sutcliffe,J.A. and Gootz,T.D. (1990). In vitro assays used to measure the activity of topoisomerases. *Anti. Agents and Chemother.* **34**: 1-7.

Bazile,S., Moreau,N., Bouzard,D. and Essiz,M. (1992). Relationships among antibacterial activity, inhibition of DNA gyrase, and intracellular accumulation of 11 fluoroquinolones. *Anti. Agents and Chemother.* **36**: 2622-2627.

Bedard,J., Wong,S. and Bryan,L.E. (1987). Accumulation of enoxacin by *Escherichia coli* and *Bacillus subtilis* . *Anti. Agents and Chemother.* **31**: 1348-1354.

Bedard,J., Chamberland,S., Wong,S., Schollaardt,T. and Bryan,L.E. (1989). Contribution of permeability and sensitivity to inhibition of DNA synthesis in determining susceptibilities of *Escherichia coli, Pseudomonas aeruginosa* and *Alcaligenes faecalis* to ciprofloxacin. *Anti. Agents and Chemother.* **33**: 1457-1464.

Benbrook,D.M. and Miller,R.V. (1986). Effects of norfloxacin on DNA metabolism in *Pseudomonas aeruginosa. Anti. Agents and Chemother.* **29**: 1-6.

Berg,D.E. (1989). Transposable elements in prokaryotes. **In:** *Gene Transfer in the Environment.* (Levy,S.B. and Miller,R.V. eds.). McGraw Hill Inc, pp99-137.

Berry,A., DeVault,J.D. and Chakrabarty,A.M. (1989). High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. *J. Bacteriol.* **171**: 2312-2317.

Bouchard,C. (1889). Influence qu'exerce sur la maladie charbonneuse l'inoculation du bacille pyocyanique. *Compt. rend.* **108**: 713. [as quoted by Gaby (1946)].

Bourguignon,G.J., Levitt,M. and Sternglanz,R. (1973). Studies on the mechanism of action of nalidixic acid. *Anti. Agents and Chemother.* **4**: 479-486.

Bryan,L.E. and Bedard,J. (1991). Impermeability to quinolones in Gram-positive and Gram-negative bacteria. *Eur. J. Clin. Microbiol. Infect. Dis.* **10**: 232-239.

Bryan,L.E., Bedard,J., Chamberland,S. and Wong,S. (1989). Quinolone antimicrobial agents: Mechanism of action and resistance development. *Clin. and Investigative Med.* **12**: 14-19.

Burman,L.G. (1977). Apparent absence of transferable resistance to nalidixic acid in pathogenic Gram-negative bacteria. *J. Anti. Chemother.* **3**: 509-516.

Cairns,J. (1963). The chromosome of *Escherichia coli. Cold Spring Harbor Symposium on Quant. Biol.* **28**: 43-46.

Celesk,R.A. and Robillard,N.J. (1989). Factors influencing the accumulation of ciprofloxacin in *Pseudomonas aeruginosa. Anti. Agents and Chemother.* **33**: 1921-1926.

Chamberland,S., Bayer,A.S., Schollaardt,T., Wong,S.A. and Bryan,L.E. (1989). Characterization of mechanisms of quinolone resistance in *Pseudomonas aeruginosa* strains isolated in vitro and in vivo during experimental endocarditis. *Anti. Agents and Chemother.* **33**: 624-634.

Chapman,J.S. and Georgopapadakou,N.H. (1988). Routes of quinolone permeation in *Escherichia coli. Anti. Agents and Chemother.* **32**: 438-442.

Chapman,J.S., Bertasso,A. and Georgopapadakou,N.H. (1989). Fleroxacin resistance in *Escherichia coli. Anti. Agents and Chemother.* **33**: 239-241.

Charrin and de Nittis. (1898). *Compt. rend.* **10**: 721. [as quoted by Jordan (1899)].

Clarke,P.H. (1989). *Pseudomonas aeruginosa* , an opportunist pathogen. **In:** *Pseudomonas Infection and Alginates: Biochemistry, Genetics and Pathology.* (Gacesa,P. and Russell,N.J. eds.).Chapman and Hall, London. pp1-12.

Cohen,S.P., Hooper,D.C., Wolfson,J.S., Souza,K.S., McMurry,L.M. and Levy,S.B. (1988). Endogenous active efflux of norfloxacin in susceptible *Escherichia coli. Anti. Agents and Chemother.* **32**: 1187-1191.

Cohen,S.P., McMurry,L.M., Hooper,D.C., Wolfson,J.S. and Levy,S.B. (1989). Cross resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: Decreased drug accumulation associated with membrane changes in addition to OmpF reduction. *Anti. Agents and Chemother.* **33**: 1318-1325.

Cook,T.M., Deitz,W.H. and Goss,W.A. (1966). Mechanism of action of nalidixic acid on *Escherichia coli* IV. Effects on the stability of cellular constituents. *J. Bact.* **91**: 774-779.

Courtright,J.B., Turowski,D.A. and Sonstein,S.A. (1988). Alteration of bacterial DNA structure, gene expression, and plasmid encoded antibiotic resistance following exposure to enoxacin. *J. Anti. Chemother.* **21 Supp B**: 1-18.

Courvalin,P. (1990). Plasmid-mediated 4-quinolone resistance: a real or apparent absence? *Anti. Agents and Chemother.* **34**: 681-684.

Cozzarelli,N.R. (1980). DNA gyrase and the supercoiling of DNA. *Science* **207**: 953-960.

Crook,S.M., Selkon,J.B. and McLardy Smith,P.D. (1985). Clinical resistance to long-term oral ciprofloxacin. *Lancet* **i**: 1275.

Crumplin,G.C., Kenwright,M. and Hirst,T. (1984). Investigations into the mechanism of action of the antibacterial agent norfloxacin. *J. Anti. Chemother.* **13 Supp B**: 9-23.

Cullen,M.E., Wyke,A.W., Kuroda,R. and Fisher,L.M. (1989). Cloning and characterization of a DNA gyrase A gene from *Escherichia coli* that confers clinical resistance to 4-quinolones. *Anti. Agents and Chemother.* **33**: 886-894.

Daikos,G.L., Lolans,V.T. and Jackson,G.G. (1988). Alterations in outer membrane proteins of *Pseudomonas aeruginosa* associated with selective resistance to quinolones. *Anti. Agents and Chemother.* **32**: 785-787.

Darveau,R.P., MacIntyre,S., Buckley,J.T. and Hancock,R.E.W. (1983). Purification and reconstitution in lipid bilayer membranes of an outer membrane pore forming protein of *Aeromonas salmonicida. J. Bact.* **156**: 1006-1011.

Davies,J. (1964). Studies on the ribosomes of streptomycin-sensitive and resistant strains of *Escherichia coli . Proc. Natl. Acad. Sci. USA.* **51**: 659-664.

Davis,B.D. and Mingioli,E.S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B12. *J. Bact.* **60**: 17-28.

Deitz,W.H., Cook,T.M. and Goss,W.A. (1966). Mechanism of action of nalidixic acid on *Escherichia coli* III. Conditions required for lethality. *J. Bact.* **91**: 768-773.

Dickie,P., Bryan,L.E. and Pickard,M.A. (1978). Effect of enzymatic adenylylation on dihydrostreptomycin accumulation in *Escherichia coli* carrying an R-factor: Model explaining aminoglycoside resistance by inactivating mechanisms. *Anti. Agents and Chemother.* **14**: 569-580.

Dimri,G.P. and Das,H.K. (1990). Cloning and sequence analysis of *gyrA* gene of *Klebsiella pneumoniae. Nucleic Acids Res.* **18**: 151-156.

Diver,J.M. and Wise,R. (1986). Morphological and biochemical changes in *Escherichia coli* after exposure to ciprofloxacin. *J. Anti. Chemother.* **18 Supp D**: 31-41.

Diver,J.M., Bryan,L.E. and Sokol,P.A. (1990a). Transformation of *Pseudomonas aeruginosa* by electroporation. *Analytical Biochem.* **189**: 75-79.

Diver,J.M., Piddock,L.J.V. and Wise,R. (1990b). The accumulation of five quinolone antibacterial agents by *Escherichia coli. J. Anti. Chemother.* **25**: 319-333.

Diver,J.M., Schollaardt,T., Rabin,H.R., Thorson,C. and Bryan,L.E. (1991). Persistence mechanisms in *Pseudomonas aeruginosa* from cystic fibrosis patients undergoing ciprofloxacin therapy. *Anti. Agents and Chemother.* **35**: 1538-1546.

Disney,G. and Dove,S. (1991). Isogenic expression of TEM derived β-lactamases. *Glaxo Group Research Ltd. Internal Report.* (manuscript in preparation).

Dorman,C.J., Barr,G.C., Bhriain,N.N. and Higgins,C.F. (1988). DNA supercoiling and the anaerobic growth phase regulation of *tonB* gene expression. *J. Bact.* **170**: 2816-2826.

Dougherty,T.J. and Saukkonen,J.J. (1985). Membrane permeability changes associated with DNA gyrase inhibitors in *Escherichia coli. Anti. Agents and Chemother.* **28**: 200-206.

Emmerich,R. and Low,O. (1899). Bakteriolytische enzyme als ursache der erworbenen immunitat und die heilung von infektionskrankheiten durch dieselben. *Z. Hyg. Infektionskrankh.* **31**: 1-15. [as quoted by Gaby (1946)].

Fasching,C.E., Tenover,F.C., Slama,T.G., Fisher,L.M., Sreedharan,S., Oram,M., Willard,K., Sinn,L.M., Gerding,D.N. and Peterson,L.R. (1991). *GyrA* mutations in ciprofloxacin-resistant, methicillin-resistant *Staphylococcus aureus* from Indiana, Minnesota, and Tennessee. *J. Infect. Dis.* **164**: 976-979.

Fisher,L.M., Mizuuchi,K., O'Dea,M.H., Ohmori,H. and Gellert,M. (1981). Site-specific interaction of DNA gyrase with DNA. *Proc. Natl. Acad. Sci. USA.* **78**: 4165-4169.

Fordos. (1860). *Compt. rend.* **li**: 215. [as quoted by Jordan (1899)].

Foster,T.J. (1983). Plasmid-determined drug resistance. *Microbiol. Rev.* **47**: 361-409.

Foulds,J. (1976). *tolF* locus in *Escherichia coli*: Chromosomal location and relationship of loci *clmB* and *tolD. J. Bact.* **128**: 604-608.

Fraenkel,E. (1917). Weitere untersuchungen uber die menschenpathogenitat des *Bacillus pyocyaneus. Z. Hyg. Infektionskrankh.* **84**: 369-423. [as quoted by Gaby (1946)].

Fujimaki,K., Fujii,T., Aoyama,H., Sato,K., Inoue,Y., Inoue,M. and Mitsuhashi,S. (1989). Quinolone resistance in clinical isolates of *Serratia marcescens. Anti. Agents and Chemother.* **33**: 785-787.

Fukuda,H., Hosaka,M., Hirai,K. and Iyobe,S. (1990). New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. *Anti. Agents and Chemother.* **34**: 1757-1761.

Gaby,W.L. (1946). A study of the dissociative behaviour of *Pseudomonas aeruginosa. J. Bact.* **51**: 217-234.

Gellert,M., Mizuuchi,K., O'Dea,M.H. and Nash,H.A. (1976). DNA gyrase : An enzyme that introduces superhelical turns into DNA. *Proc. Natl. Acad. Sci. USA* **73**: 3872-3876.

Gellert,M., Mizuuchi,K., O'Dea,M.H., Itoh,T. and Tomizawa,J. (1977). Nalidixic acid resistance: A second genetic character involved in DNA gyrase activity. *Proc. Natl. Acad. Sci. USA.* **74**: 4772-4776.

Gessard. (1882). La pyocyanine, These. Paris. [as quoted by Jordan (1899)].

Gootz,T.D. and Martin,B.A. (1991). Characterization of high-level quinolone resistance in *Campylobacter jejuni. Anti Agents and Chemother.* **35**: 840-845.

Goss,W.A., Deitz,W.H. and Cook,T.M. (1964). Mechanism of action of nalidixic acid on *Escherichia coli. J. Bact.* **88**: 1112-1118.

Goss,W.A., Deitz,W.H. and Cook,T.M. (1965). Mechanism of action of nalidixic acid on *Escherichia coli* II. Inhibition of deoxyribonucleic acid synthesis. *J. Bact.* **89**: 1068-1074.

Goswitz,J.J., Willard,K.E., Fasching,C.E. and Peterson,L.R. (1992). Detection of *gyrA* gene mutations associated with ciprofloxacin resistance in methicillin-resistant *Staphylococcus aureus* : Analysis by polymerase chain reaction and automated direct DNA sequencing. *Anti. Agents and Chemother.* **36**: 1166-1169.

Govan,J.R.W. (1988). Alginate biosynthesis and other unusual characteristics associated with the pathogenesis of *Pseudomonas aeruginosa* in cystic fibrosis. **In:** *Bacterial Infections of Respiratory and Gastrointestinal Mucosae.* (Donachie,W., Griffiths,E. and Stephen,J. eds.). I.R.L. Press, Oxford. pp67-96.

Govan,J.R.W., Fyfe,J.A.M. and Baker,N.K. (1983). Heterogeneity and reduction in pulmonary clearance of mucoid *Pseudomonas aeruginosa. Rev. Infect. Dis.* **5**: S874-879.

Gutmann,L., Williamson,R., Moreau,N., Kitzis,M-D., Collatz,E., Acar,J.F. and Goldstein,F.W. (1985). Cross-resistance to nalidixic acid, trimethoprim, and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella, Enterobacter*, and *Serratia. J. Infect. Dis.* **151**: 501-507.

Hancock,R.E.W., Siehnel,R. and Martin,N. (1990). Outer membrane proteins of Pseudomonas. *Mol. Microbiol.* **4**: 1069-1075.

Hane,M.W. and Wood,T.H. (1969). *Escherichia coli* K-12 mutants resistant to nalidixic acid: Genetic mapping and dominance studies. *J. Bact.* **99**: 238-241.

Hallett,P. and Maxwell,A. (1991). Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein; enzymatic analysis of the mutant proteins. *Anti. Agents and Chemother.* **35**: 335-340.

Harder,K.J., Nikaido,H. and Matsuhashi,M. (1981). Mutants of *Escherichia coli* that are resistant to certain beta-lactam compounds lack the *ompF* porin. *Anti. Agents and Chemother.* **20**: 549-552.

Hashmi,Z.S. and Smith,J.M.B. (1991). Outer membrane changes in quinolone resistant *Pseudomonas aeruginosa. J. Anti. Chemother.* **28**: 465-470.

Hedges,R.W. and Jacob,A.E. (1974). Transposition of ampicillin resistance from RP4 to other replicons. *Mol. Gen. Genet.* **132**: 31-40.

Heisig,P. and Wiedemann,B. (1991). Use of a broad-host-range *gyrA* plasmid for genetic characterization of fluoroquinolone-resistant Gram-negative bacteria. *Anti. Agents and Chemother.* **35**: 2031-2036.

Heisig,P., Schedletzky,H. and Falkenstein-Paul,H. (1993). Mutations in the *gyrA* gene of a highly fluoroquinolone-resistant clinical isolate of *Escherichia coli. Anti. Agents and Chemother.* **37**: 696-701.

Higgins,N.P. and Cozzarelli,N.R. (1982). The binding of gyrase to DNA: Analysis by retention by nitrocellulose filters. *Nucleic Acids Res.* **10**: 6833-6839.

Hirai,K., Aoyama,H., Irakura,T., Iyobe,S. and Mitsuhashi,S. (1986a). Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli. Anti. Agents and Chemother.* **29**: 535-538.

Hirai,K., Aoyama,H., Suzue,S., Irikura,T., Iyobe,S. and Mitsuhashi,S. (1986b). Isolation and characterization of norfloxacin-resistant mutants of *Escherichia coli* K12. *Anti. Agents and Chemother.* **30**: 248-253.

Hirai,K., Suzue,S., Irikura,T., Iyobe,S. and Mitsuhashi,S. (1987). Mutations producing resistance to norfloxacin in *Pseudomonas aeruginosa. Anti. Agents and Chemother.* **31**: 582-586.

Holmes,M.L. and Dyall-Smith,M.L. (1991). Mutations in DNA gyrase result in novobiocin resistance in halophilic archaebacteria. *J. Bact.* **173**: 642-648.

Hooper,D.C., Wolfson,J.S., Souza,K.S., Ng,E.Y., McHugh,G.L. and Swartz,M.N. (1989). Mechanisms of quinolone resistance in *Escherichia coli*: Characterization of *nfxB* and *cfxB,* two mutant resistance loci decreasing norfloxacin accumulation. *Anti. Agents and Chemother.* **33**: 283-290.

Hopewell,R., Oram,M., Briesewitz,R. and Fisher,L.M. (1990). DNA cloning and organization of the *Staphylococcus aureus gyrA* and *gyrB* genes: Close homology among gyrase proteins and implications for 4-quinolone action and resistance. *J. Bact.* **172**: 3481-3484.

Horowitz,D.S. and Wang,J.C. (1987). Mapping the active site tyrosine of *Escherichia coli* DNA gyrase. *J. Biol. Chem.* **262**: 5339-5344.

Inoue,S., Ohue,T., Yamagishi,J., Nakamura,S. and Shimizu,M. (1978). Mode of incomplete cross-resistance among pipemidic, piromidic and nalidixic acids. *Anti. Agents and Chemother.* **14**: 240-245.

Inoue,Y., Sato,K., Fujii,T., Hirai,K., Inoue,M., Iyobe,S. and Mitsuhashi,S. (1987). Some properties of subunits of DNA gyrase from *Pseudomonas aeruginosa* PAO1 and its nalidixic acid-resistant mutant. *J. Bact.* **169**: 2322-2325.

Iordanescu,S., Surdeanu,M., Della Latta,P. and Novick,R.P. (1978). Incompatibility and molecular relationships between small staphylococcal plasmids carrying the same resistance marker. *Plasmid* **1**: 468-479.

Ishii,H., Sato,K., Hoshino,K., Sato,M., Yamaguchi,A., Sawai,T. and Osada,Y. (1991). Active efflux of ofloxacin by a highly quinolone-resistant strain of *Proteus vulgaris. J. Anti. Chemother.* **28**: 827-836.

Jacoby,G.A. (1986). Resistance plasmids of *Pseudomonas.* **In:***The Bacteria Vol. X: The Biology of Pseudomonas.* (Sokatch,J.R. ed.). Academic Press, New York. pp265-293.

Jin,Y.F. and Piddock,L.J.V. (1993). Characterisation of the mechanism of ciprofloxacin-resistance in laboratory mutants of *Streptococcus pneumoniae.* **In:** Program and Abstracts of the 6th ECSMID, Seville, Spain. Abs. 1039 p244.

Jordan,E.O. (1899). *Bacillus pyocyaneus* and its pigments. *J. Exp. Med.* **4**: 627-647.

Kaatz,G.W. and Seo,S.M. (1988). Mechanism of ciprofloxacin resistance in *Pseudomonas aeruginosa. J. Infect. Dis.* **158**: 537-541.

Kaatz,G.W., Seo,S.M. and Ruble,C.A. (1991). Mechanisms of fluoroquinolone resistance in *Staphylococcus aureus. J. Infect. Dis.* **163**: 1080-1086.

Kaatz,G.W., Seo,S.M. and Ruble,C.A. (1993). Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus. Anti. Agents and Chemother.* **37**: 1086-1094.

Kato,N., Miyauchi,M., Muto,Y., Watanabe,K. and Ueno,K. (1988). Emergence of fluoroquinolone resistance in *Bacteroides fragilis* accompanied by resistance to β-lactam antibiotics. *Anti . Agents and Chemother.* **32**: 1437-1438.

Klevan,L. and Wang,J.C. (1980). Deoxyribonucleic acid gyrase-deoxyribonucleic acid complex containing 140 base copies of deoxyribonucleic acid and an $\alpha_2\beta_2$ protein core. *Biochem.* **19**: 5229-5234.

Koga,H., Itoh,A., Murayama,S., Suzue,S. and Irikura,T. (1980). Structure-activity relationships of antibacterial 6,7- and 7,8-disubstituted 1-alkyl-1,4-dihydro-4-oxoquinoline-3-carboxylic acids. *J. Med. Chem.* **23**: 1358-1363.

Kotera,Y., Watanabe,M., Yoshida,S., Inoue,M. and Mitsuhashi,S. (1991). Factors influencing the uptake of norfloxacin by *Escherichia coli. J. Anti. Chemother.* **27**: 733-739.

Krueger,S., Zaccai,G., Wlodawer,A., Langowski,J., O'Dea,M., Maxwell,A. and Gellert,M. (1990). Neutron and light-scattering studies of DNA gyrase and its complex with DNA. *J. Mol. Biol.* **211**: 211-220.

Lai,C.J. and Weisblum,B. (1971). Altered methylation of ribosomal RNA in an erythromycin-resistant strain of *Staphylococcus aureus. Proc. Natl. Acad. Sci. USA.* **68**: 856-860.

Lampe,M.F. and Bott,K.F. (1984). Cloning the *gyrA* gene of *Bacillus subtilis. Nucleic Acids Res.* **12**: 6307-6323.

Lartigau,A.J. (1898). A contribution to the study of pathogenesis of the *Bacillus pyocyaneus*, with special reference to its relation to an epidemic of dysentery. *J. Exptl. Med.* **3**: 595-609.

Legakis,N.J., Tzouvelekis,L.S., Makris,A. and Kotsifaki,H. (1989). Outer membrane alterations in multiresistant mutants of *Pseudomonas aeruginosa* selected by ciprofloxacin. *Anti. Agents and Chemother.* **33**: 124-127.

Le Goffic,F. (1985). Les quinolones, méchanismes d'action. **In:** *Les Nouvelles Quinolones.* (Pocidalo,J.J., Vachon,F. and Regmer,B. eds.). Edition Arnette, Paris. pp15-23.

Lesher,G.Y., Froelich,E.J., Gruett,M.D., Bailey,J.H. and Brundage,R.P. (1962). 1,8-Naphthyridine derivatives. A new class of chemotherapeutic agents. *J. Med. Pharm. Chem.* **5**: 1063-1065.

Levy,S.B., McMurry,L., Onigman,P. and Saunders,R.M. (1977). Plasmid-mediated tetracycline resistance in *Escherichia coli.* **In:** *R-factors: Their Properties and Possible Control.* (Drews,J. and Hogenauer eds.). Springer-Verlag, Vienna. pp181-203.

Lewin,C.S. and Hastings,T.S. (1990). In vitro activities of oxolinic acid, ciprofloxacin and norfloxacin against *Aeromonas salmonicida. J. Fish Dis.* **13**: 377-384.

Lewin,C.S. and Smith,J.T. (1987). Ciprofloxacin does not exhibit mechanism B against *Staphylococcus albus. J. Pharm. Pharmacol.* **39 Supp**: 21P

Lewin,C.S., Amyes,S.G.B. and Smith,J.T. (1989a). Bactericidal activity of enoxacin and lomefloxacin against *Escherichia coli* KL16. *Eur. J. Clin. Microbiol.* **8**: 731-733.

Lewin,C.S., Morrissey,I. and Smith,J.T. (1989b). Role of oxygen in the bactericidal action of the 4-quinolones. *Rev. Infect. Dis.* **11 Supp 5**: 913-914.

Lewin,C.S., Howard,B.M.A., Ratcliffe,N.T. and Smith,J.T.(1989c) 4-quinolones and the SOS response. *J. Med. Microbiol.* **29**: 139-144.

Lewin,C.S., Allen,R.A. and Amyes,S.G.B. (1990). Potential mechanisms of resistance to the modern fluorinated 4-quinolones. *J. Med. Microbiol.* **31**: 153-161.

Lewis,M.J. (1989). Mechanisms of resistance. **In:** *Antimicrobial Chemotherapy 2nd Edition.* (Greenwood,D. ed.). Oxford University Press, Oxford. pp136-145.

Liu,P.V. (1979). Toxins of *Pseudomonas aeruginosa.* **In:** *Pseudomonas aeruginosa: Clinical Manifestations of Infection and Current Therapy.* (Doggett,R.G. ed.). Academic Press, London. pp63-88.

Lowbury,E.J.L. (1975). Biological importance of *Pseudomonas aeruginosa:* medical aspects. **In:** *Genetics and Biochemistry of Pseudomonas.* (Clarke,P.H. and Richmond,M.H. eds.).Wiley, Chichester. pp37-65.

Masuda,N. and Ohya,S. (1992). Cross-resistance to meropenem, cephems and quinolones in *Pseudomonas aeruginosa. Anti. Agents and Chemother.* **36**: 1847-1851.

Matthew,M., Harris,A.M., Marshall,M.J. and Ross,G.W. (1975). The use of analytical isoelectric focusing for detection and identification of β-lactamases. *J. Gen. Microbiol.* **88**: 169-178.

McMurry,L., Petrucci Jr.,R.E. and Levy,S.B. (1980). Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli. Proc. Natl. Acad. Sci. USA.* **77**: 3974-3977.

Menzel,R. and Gellert,M. (1983). Regulation of the genes for *E.coli* DNA gyrase: Homeostatic control of DNA supercoiling. *Cell* **34**: 105-113.

Michéa-Hamzehpour,M., Furet,Y.X. and Pechère,J-C. (1991). Role of protein D2 and lipopolysaccharide in diffusion of quinolones through the outer membrane of *Pseudomonas aeruginosa. Anti. Agents and Chemother.* **35**: 2091-2097.

Minami,S., Yotsuji,A., Inoue,M. and Mitsuhashi,S. (1980). Induction of β-lactamase by various β-lactam antibiotics in *Enterobacter cloacae. Anti. Agents and Chemother.* **18**: 382-385.

Moniot-Ville,N., Guibert,J., Moreau,N., Acar,J.F., Collatz,E. and Gutmann,L. (1991). Mechanisms of quinolone resistance in a clinical isolate of *Escherichia coli* highly resistant to fluoroquinolones but susceptible to nalidixic acid. *Anti. Agents and Chemother.* **35**: 519-523.

Moorhead,P.J. and Parry,H.E. (1965). Treatment of sonne dysentery. *Br. Med. J.* **2**: 913-915.

Morrison,A. and Cozzarelli,N.R. (1981). Contacts between DNA gyrase and its binding site on DNA: Features of symmetry and asymmetry revealed by protection from nucleases. *Proc. Natl. Acad. Sci. USA.* **78**: 1416-1420.

Morrissey,I., Lewin,C.S. and Smith,J.T. (1990). The influence of oxygen upon bacterial potency. **In:** *The 4-Quinolones: Antibacterial Agents in vitro.* (Crumplin,G.C. ed.). Springer-Verlag, London. pp23-36.

Munshi,M.H., Haider,K., Rahaman,M.M., Sack,D.A., Ahmed,Z.U. and Morshed,M.G. (1987). Plasmid-mediated resistance to nalidixic acid in *Shigella dysenteriae* type 1. *Lancet* **ii**: 419-421.

Nakamura,S., Inoue,S., Shimizu,M., Iyobe,S. and Mitsuhashi,S. (1976). Inhibition of conjugal transfer of R plasmids by pipemidic acid and related compounds. *Anti. Agents and Chemother.* **10**: 779-785.

Nakanishi,N., Yoshida,S., Wakebe,H., Inoue,M. and Mitsuhashi,S. (1991a). Mechanisms of clinical resistance to fluoroquinolones in *Enterococcus faecalis. Anti. Agents and Chemother.* **35**: 1053-1059.

Nakanishi,N., Yoshida,S., Wakebe,H., Inoue,M., Yamaguchi,T. and Mitsuhashi,S. (1991b). Mechanisms of clinical resistance to fluoroquinolones in *Staphylococcus aureus. Anti. Agents and Chemother.* **35**: 2562-2567.

Neyfakh,A.A., Borsch,C.M. and Kaatz,G.W. (1993). Fluoroquinolone resistance protein *norA* of *Staphylococcus aureus* is a multidrug efflux transporter. *Anti. Agents and Chemother.* **37**: 128-129.

Nicas,T.I. and Hancock,R.E.W. (1983). *Pseudomonas aeruginosa* outer membrane permeability: Isolation of a porin protein F-deficient mutant. *J. Bact.* **153**: 281-285.

Nikaido,H. and Hancock,R.E.W. (1986). Outer membrane permeability of *Pseudomonas aeruginosa.* **In:** *The Bacteria. Vol. X: The Biology of Pseudomonas.* (Sokatch,J.R. ed.). Academic Press, New York. pp145-193.

Oram,M. and Fisher,L.M. (1991). 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the Polymerase Chain Reaction. *Anti. Agents and Chemother.* **35**: 387-389.

Orr,E. and Staudenbauer,W.L. (1982). *Bacillus subtilis* DNA gyrase: Purification of subunits and reconstitution of supercoiling activity. *J. Bact.* **151**: 524-527.

Palleroni,N.J. (1986). Taxonomy of the pseudomonads. **In**: *The Bacteria. Vol. X: The Biology of Pseudomonas.* (Sokatch,J.R. ed.). Academic Press, New York. pp3-25.

Palleroni,N.J., Kunisawa,R., Contopoulou,R. and Doudoroff,M. (1973). Nucleic acid homologies in the genus *Pseudomonas. Int. J. Syst. Bact.* **23**: 333-339.

Percival,A. (1991). Impact of chemical structure on quinolone potency, spectrum and side effects. *J. Anti. Chemother.* **28 Supp C**: 1-8.

Phillips,I., Culebras,E., Moreno,F. and Baquero,F. (1987). Induction of the SOS response by new 4-quinolones. *J. Anti. Chemother.* **20**: 631-638.

Piddock,L.J.V. (1991). Mechanism of quinolone uptake into bacterial cells. *J. Anti Chemother.* **27**: 399-403.

Piddock,L.J.V. and Wise,R. (1987). Induction of the SOS response in *Escherichia coli* by the 4-quinolone antimicrobial agents. *FEMS. Microbiol. Lett.* **41**: 289-294.

Piddock,L.J.V. and Zhu,M. (1991). Mechanism of action of sparfloxacin against and mechanism of resistance in Gram-negative and Gram-positive bacteria. *Anti. Agents and Chemother.* **35**: 2423-2427.

Piddock,L.J.V., Walters,R.N. and Diver,J.M. (1990). Correlation of quinolone MIC and inhibition of DNA, RNA and protein synthesis and induction of the SOS response in *Escherichia coli. Anti. Agents and Chemother.* **34**: 2331-2336.

Piddock,L.J.V., Wijnands,W.J.A. and Wise,R. (1987). Quinolone / ureidopenicillin cross-resistance. *Lancet* **ii**: 907.

Piddock,L.J.V., Panchal,S., Inglis,T.J.J. and Hawkey,P.M. (1992). High-level quinolone resistance in *Pseudomonas aeruginosa. J. Anti. Chemother.* **30**: 229-231.

Platt,D.J. and Black,A.C. (1987). Plasmid ecology and the elimination of plasmids by 4-quinolones. *J. Anti. Chemother.* **20**: 137-142.

Pollack,M. (1984). The virulence of *Pseudomonas aeruginosa. Rev. Infect. Dis.* **6**: S617-626.

Power,E.G.M., Muñoz Bellido,J.L. and Phillips,I. (1992). Detection of ciprofloxacin resistance in Gram-negative bacteria due to alterations in *gyrA. J. Anti. Chemother.* **29**: 9-17.

Rådberg,G., Nilsson,L.E. and Svensson,S. (1990). Development of quinolone-imipenem cross resistance in *Pseudomonas aeruginosa* during exposure to ciprofloxacin. *Anti. Agents and Chemother.* **34**: 2142-2147.

Ratcliffe,N.T. and Smith,J.T. (1985). Norfloxacin has a novel bactericidal mechanism unrelated to that of other 4-quinolones. *J. Pharm. Pharmacol.* **37 Supp**: 92P.

Rau,D.C., Gellert,M., Thoma,F. and Maxwell,A. (1987). Structure of the DNA gyrase-DNA complex as revealed by transient electric dichroism. *J. Mol. Biol.* **193**: 555-569.

Reece,R.J. and Maxwell,A. (1991). DNA gyrase: Structure and function. *Crit. Rev. Biochem. Mol. Biol.* **26**: 335-375.

Reid,A.J., Simpson,I.N., Harper,P.B. and Amyes,S.G.B. (1988). Cephaloridine resistance in Gram-negative bacteria isolated in Scotland. *J. Pharm. Pharmacol.* **40**: 571-573.

Rella,M. and Haas,D. (1982). Resistance of *Pseudomonas aeruginosa* PAO to nalidixic acid and low levels of β-lactam antibiotics: Mapping of chromosomal genes. *Anti. Agents and Chemother.* **22**: 242-249.

Reynolds,A.V. and Smith,J.T. (1979). Enzymes which modify aminoglycoside antibiotics. **In:** *Recent Advances in Infection.* (Reeves,D.S. and Geddes,A. eds.). Churchill Livingstone, Edinburgh. pp165-181.

Roberts,C.M., Batten,J. and Hodson,M.E. (1985). Ciprofloxacin-resistant *Pseudomonas. Lancet* **i**: 1442.

Robillard,N.J. (1990). Broad-host-range gyrase A gene probe. *Anti. Agents and Chemother.* **34**: 1889-1894.

Robillard,N.J. and Scarpa,A.L. (1988). Genetic and physiological characterization of ciprofloxacin resistance in *Pseudomonas aeruginosa* PAO. *Anti. Agents and Chemother.* **32**: 535-539.

Rohner,P., Peebo,M., Lew,D.P., Auckenthaler,R. and Pechère,J-C. (1992). Comparative in-vitro activity of new quinolones against clinical isolates and resistant mutants. *J. Anti. Chemother.* **29**: 41-48.

Rose,K.M. (1988). DNA topoisomerases as targets for chemotherapy. *FASEB J.* **2**: 2474-2478.

Roy,C., Foz,A., Segura,C., Tirado,M., Fuster,C. and Reig,R. (1983). Plasmid-determined β-lactamases identified in a group of 204 ampicillin-resistant Enterobacteriaceae. *J. Anti. Chemother.* **12**: 507-510.

Rustige,C., Chu,D.T.W., Sörgel,F. and Wiedemann,B. (1990). New aspects on structure-activity relationships of quinolones. **In**: Program and Abstracts of the 13th ICAAC, Atlanta USA. Abs. 401 p153.

Ryan,M.J. (1976). Coumermycin A: A preferential inhibitor of replicative DNA synthesis in *Escherichia coli* I. In vivo characterization. *Biochem.* **15**: 3769-3777.

Sabath,L.D. and Abraham,E.P. (1964). Synergistic action of penicillins and cephalosporins against *Pseudomonas pyocyanea. Nature* **204**: 1066-1069.

Sanders,C.C., Sanders Jr.,W.E., Goering,R.V. and Werner,V. (1984). Selection of multiple antibiotic resistance by quinolones, β-lactams, and aminoglycosides with special reference to cross-resistance between unrelated drug classes. *Anti. Agents and Chemother.* **26**: 797-801.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74**: 5463-5467.

Sato,K., Inoue,Y., Fujii,T., Aoyama,H., Inoue,M. and Mitsuhashi,S. (1986). Purification and properties of DNA gyrase from a fluoroquinolone-resistant strain of *Escherichia coli. Anti. Agents and Chemother.* **30**: 777-780.

Schentag,J.J. and Domagala,J.M. (1985). Structure-activity relatioships with the quinolone antibiotics. *Res. Clin. Forums.* **7 (2)**: 9-13.

Seleen,W.A. and Stark,C.N. (1943). Some characteristics of green-fluorescent pigment-producing bacteria. *J. Bact.* **46**: 491-500.

Shannon,K. and Phillips,I. (1982). Mechanisms of resistance to aminoglycosides in clinical isolates. *J. Anti. Chemother.* **9**: 91-102.

Shaw,W.V. (1967). The enzymatic acetylation of chloramphenicol by extracts of R factor-resistant *Escherichia coli. J. Biol. Chem.* **242**: 687-693.

Shaw,W.V. (1971). Comparative enzymology of chloramphenicol resistance. *Ann. N.Y. Acad. Sci.* **182**: 234-242.

Shaw,W.V. (1983). Chloramphenicol acetyltransferase: Enzymology and molecular biology. *Crit. Rev. Biochem.* **14**: 1-46.

Shen,L.L. and Pernet,A.G. (1985). Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: The target of the drugs is DNA. *Proc. Natl. Acad. Sci. USA.* **82**: 307-311.

Shen,L.L., Mitscher,L.A., Sharma,P.N., O'Donnell,T.J., Chu,D.W.T., Cooper,C.S., Rosen,T. and Pernet,A.G. (1989). Mechanism of inhibition of DNA gyrase by quinolone antibacterials: A cooperative drug-DNA binding model. *Biochem.* **28**: 3886-3894.

Simpson,I.N., Knothe,H., Plested,S.J. and Harper,P.B. (1986). Qualitative and quantitative aspects of β-lactamase production as mechanisms of β-lactam resistance in a survey of clinical isolates from faecal samples. *J. Anti. Chemother.* **17**: 725-737.

Sköld,O. (1976). R-factor-mediated resistance to sulfonamides by a plasmid-borne, drug-resistant dihydropteroate synthase. *Anti. Agents and Chemother.* **9**: 49-54.

Smith,J.T. (1984a). Awakening the slumbering potential of the 4-quinolone antibacterials. *Pharm. J.* **233**: 299-305.

Smith,J.T. (1984b). Mutational resistance to the 4-quinolone antimicrobial agents. *Eur. J. Clin. Microbiol.* **3**: 347-350.

Smith,J.T. and Lewin,C.S. (1988). Chemistry and mechanisms of action of the quinolone antibacterials. **In:** *The Quinolones.* (Andriole,V.T. ed.) Academic Press, London. pp23-82.

Soussy,C.J., Wolfson,J.S., Ng,E.Y. and Hooper,D.C. (1993). Limitations of the plasmid complementation test for determination of quinolone resistance (QR) due to changes in the gyrase A protein and identification of a conditional locus. **In:** Program and Abstracts of the 33rd ICAAC, New Orleans, USA. Abs. 1097 pp 320.

Spratt,B.G. (1983). Penicillin-binding proteins and the future of β-lactam antibiotics. *J. Gen. Microbiol.* **129**: 1247-1260.

Sreedharan,S., Oram,M., Jensen,B., Peterson,L.R. and Fisher,L.M. (1990). DNA gyrase *gyrA* mutations in ciprofloxacin-resistant strains of *Staphylococcus aureus*: Close similarity with quinolone resistance mutations in *Escherichia coli. J. Bact.* **172**: 7260-7262.

Sreedharan,S., Peterson,L.R. and Fisher,L.M. (1991). Ciprofloxacin resistance in coagulase-positive and -negative Staphylococci: Role of mutations at serine 84 in the DNA gyrase A protein of *Staphylococcus aureus* and *Staphylococcus epidermidis. Anti. Agents and Chemother.* **35**: 2151-2154.

Staudenbauer,W.L. and Orr,E. (1981). DNA gyrase: Affinity chromatography on novobiocin-sepharose and catalytic properties. *Nucleic Acids Res.* **9**: 3589-3603.

Sugino,A., Higgins,N.P. and Cozzarelli,N.R. (1980). DNA gyrase subunit stoichiometry and the covalent attachment of subunit A to DNA during DNA cleavage. *Nucleic Acids Res.* **8**: 3865-3874.

Sugino,A., Peebles,C.L., Kreuzer,K.N. and Cozzarelli,N.R. (1977). Mechanism of action of nalidixic acid: Purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. *Proc. Natl. Acad. Sci. USA.* **74**: 4767-4771.

Sutcliffe,J.A., Gootz,T.D. and Barrett,J.F. (1989). Biochemical characteristics and physiological significance of major DNA topoisomerases. *Anti. Agents and Chemother.* **33**: 2027-2033.

Swanberg,S.L. and Wang,J.C. (1987). Cloning and sequencing of the *Escherichia coli gyrA* gene coding for the A subunit of DNA gyrase. *J. Mol. Biol.* **197**: 729-736.

Swedberg,G. and Sköld,O. (1980). Characterization of different plasmid-borne dihydropteroate synthases mediating bacterial resistance to sulfonamides. *J. Bact.* **142**: 1-7.

Takahashi,S and Nagano,Y. (1984). Rapid procedure for the isolation of plasmid DNA and application to epidemiological analysis. *J. Clin. Microbiol.* **20**: 608-613.

Takiff,H., Guerrero,C., Philipp,W., Bodmer,T., Jacobs,W.R., Cole,S.T. and Telenti,A. (1993). Mutations in the gyrase A gene of *Mycobacterium tuberculosis* confer resistance to fluoroquinolones. **In:** Program and Abstracts of the 33rd ICAAC, New Orleans, USA. Abs. 1092 pp 320.

Tanaka,M., Sato,K., Kimura,Y., Hayakawa,I., Osada,Y. and Nishino,T. (1991). Inhibition by quinolones of DNA gyrase from *Staphylococcus aureus. Anti. Agents and Chemother.* **35**: 1489-1491.

Teraoka,H. and Tanaka,K. (1974). Properties of ribosomes from *Streptomyces erythreus* and *Streptomyces griseus. J. Bact.* **120**: 316-321.

Thiara,A.S. and Cundliffe,E. (1988). Cloning and characterization of a DNA gyrase B gene from *Streptomyces sphaeroides* that confers resistance to novobiocin. *EMBO. J.* **7**: 2255-2259.

Thomson,K.S., Sanders,C.C. and Hayden,M.E. (1991). In vitro studies with five quinolones: Evidence for changes in relative potency as quinolone resistance rises. *Anti. Agents and Chemother.* **35**: 2329-2334.

Thumm. (1895). Beiträge zur biologie der fluorescierenden bakterien. *Arb. Bact. Inst. Tech. Hochshule zu Karlsruhe* **1**: 291. [as quoted by Jordan (1899)].

Ubukata,K., Itoh-Yamashita,N. and Konno,M. (1989). Cloning and expression of the *norA* gene for fluoroquinolone resistance in *Staphylococcus aureus. Anti. Agents and Chemother.* **33**: 1535-1539.

Waddell,W.J. (1956). A simple ultraviolet spectrophotometric method for the determination of protein. *J. Lab. Clin. Med.* **48**: 311-314.

Walters,R.N., Piddock,L.J.V. and Wise,R. (1989). The effect of mutations in the SOS response on the kinetics of quinolone killing. *J. Anti. Chemother.* **24**: 863-873.

Wang,J.C. (1974). Interactions between twisted DNAs and enzymes: The effects of superhelical turns. *J. Mol. Biol.* **87**: 797-816.

Wang,Y., Huang,W.M. and Taylor,D.E. (1993). Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutations. *Anti. Agents and Chemother.* **37**: 457-463.

Weisser,J. and Wiedemann,B. (1985). Elimination of plasmids by new 4-quinolones. *Anti. Agents and Chemother.* **28**:700-702.

Weisser,J. and Wiedemann,B. (1986). Elimination of plasmids by enoxacin and ofloxacin at near inhibitory concentrations. *J. Anti. Chemother.* **18**: 575-583.

Weisser,J. and Wiedemann,B. (1987). Inhibition of R-plasmid transfer in *Escherichia coli* by 4-quinolones. *Anti. Agents and Chemother.* **31**: 531-534.

Wiedemann,B. (1989). The epidemiology of β-lactamases. *J. Anti. Chemother.* **2 Supp B**: 1-22.

Willmott,C.J.R. and Maxwell,A. (1993). A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. *Anti. Agents and Chemother.* **37**: 126-127.

Winship,P.R. (1989). An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. *Nucleic Acids Res.* **17**: 1266.

Wolfson,J.S. and Hooper,D.C. (1985). The fluoroquinolones: Structures, mechanisms of action and resistance, and spectra of activity in vitro. *Anti. Agents and Chemother.* **28**: 581-586.

Wolfson,J.S. and Hooper,D.C. (1989). Bacterial resistance to quinolones: Mechanisms and clinical importance. *Rev. Infect. Dis:* **11 Supp 5**: S960-968.

Worcel,A. (1974). Studies on the folded chromosome of *Escherichia coli.* **In:** *Mechanism and Regulation of DNA Replication.* (Kolber,A.R. and Kohiyama,M. eds.). Plenum Press, London. pp201-224.

Working Party on Antibiotic Sensitivity Testing of the British Society for Antimicrobial Chemotherapy. (1991). *J. Anti. Chemother.* **27 Supp D.**

Yamagishi,J., Yoshida,H., Yamayoshi,M. and Nakamura,S. (1986). Nalidixic acid-resistant mutations of the *gyrB* gene of *Escherichia coli. Mol. Gen. Genet.* **204**: 367-373.

Yamagishi,J., Furutani,Y., Inoue,S., Ohue,T., Nakamura,S. and Shimizu,M. (1981). New nalidixic acid resistance mutations related to deoxyribonucleic acid gyrase activity. *J. Bact.* **148**: 450-458.

Yamamoto,N. and Droffner,M.L. (1985). Mechanisms determining aerobic or anaerobic growth in the facultative anaerobe *Salmonella typhimurium. Proc. Natl. Acad. Sci. USA.* **82**: 2077-2081.

Yamashita,S., Inone,Y., Sato,K., Inone,M. and Mitsuhashi,S. (1986). DNA gyrase from fluoroquinolone resistant *Enterobacter cloacae. Proc. 1st Int. Symp. New Quinolones* **13**.

Yoshida,H., Kojima,T., Yamagishi,J. and Nakamura,S. (1988). Quinolone-resistant mutations of the *gyrA* gene of *Escherichia coli. Mol. Gen. Genet.* **211**: 1-7.

Yoshida,H., Bogaki,M., Nakamura,M. and Nakamura,S. (1990a). Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli. Anti. Agents and Chemother.* **34:** 1271-1272.

Yoshida,H., Nakamura,M., Bogaki,M. and Nakamura,S. (1990b). Proportion of DNA gyrase mutants among quinolone-resistant strains of *Pseudomonas aeruginosa. Anti. Agents and Chemother.* **34**: 1273-1275.

Yoshida,H., Bogaki,M., Nakamura,S., Ubukata,K. and Konno,M. (1990c). Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. *J. Bact.* **172**: 6942-6949.

Yoshida,H., Bogaki,M., Nakamura,M., Yamanaka,L.M. and Nakamura,S. (1991). Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli. Anti. Agents and Chemother.* **35**: 1647-1650.

Yoshida,H., Nakamura,M., Bogaki,M., Ito,H., Kojima,T., Hattori,H. and Nakamura,S. (1993). Mechanism of action of quinolones against *Escherichia coli* DNA gyrase. *Anti. Agents and Chemother.* **37**: 839-845.

Young,M. and Hancock,R.E.W. (1992). Fluoroquinolone supersusceptibility mediated by outer membrane protein OprH overexpression in *Pseudomonas aeruginosa*: Evidence for involvement of a nonporin pathway. *Anti. Agents and Chemother.* **36**: 2365-2369.

Zyskind,J.W. and Bernstein,S.I. (1989). Isolation and purification of *E.coli* chromosomal DNA. **In:** *Recombinant DNA Laboratory Manual.* (Zyskind,J.W. and Bernstein,S.I. eds.). Academic Press Inc., London. pp12-15.

APPENDIX.

Appendix i : Pyocin types of mutant isolates

Appendix ii : Integral values from the densitometer readings used to calculate the IC_{50} values for ciprofloxacin, ofloxacin, norfloxacin and novobiocin.

APPENDIX i.

STRAIN	PYOCIN TYPE
U423	76 / ut (s - 7, a, d, e)
U423A	ut / ut (s - 7)
U423B	ut / ut (s - 7)
U423C	9 / ut (s - 7)
U423D	9 / ut (s - 7)
U423E	ut / ut (s - 7, a)
U423F	ut / ut (s - 7)
91-32	1 / d (s - a)
91-32-1	3 / c
91-32-2	4 / d
91-32-3	10 / d
91-32-4	3 / x
91-32-5	1 / d
91-32-6	3 / k
8252	10 / ut (s - 5, a, d, e)
3221	10 / ut (s - a, d, e)

ut - untypable strains, s - sensitivity shown to reagents named.

APPENDIX ii.

STRAIN	DRUG	INTEGRAL AT DRUG CONCENTRATION (mg/l) OF :-						IC_{50} (mg/l)
		0	0.25	0.5	1	2	4	
PAO1	CPX	1251	-	904	584	-	-	0.94
	OFX	1688	-	-	1122	1024	894	4
	NFX	1204	1187	1145	1027	551	-	1.89
	NOV	1053	1267	1103	931	157	-	1.52

		0	0.25	0.5	1			
PAO1	CPX	4239	2660	1628	104			0.38
Colindale	OFX	4254	4298	3523	1505			0.85
	NFX	4896	3603	2944	1153			0.64
	NOV	3040	1767	1679	222			0.55

		0	0.25	0.5	64	128	256	
C1	CPX	2097	-	-	2041	1297	661	178.0
	OFX	2087	-	-	1439	468	-	90.1
	NFX	1690	-	-	1264	1089	761	223.3
	NOV	2081	1471	1037	-	-	-	0.49

		0	0.25	16	32	64	128	
C48	CPX	2772	-	1707	743	-	-	18.7
	OFX	10630	-	6212	4837	-	-	27.3
	NFX	4189	-	-	3202	2065	-	63.2
	NOV	6702	3279	-	-	-	-	0.24

STRAIN	DRUG	INTEGRAL AT DRUG CONCENTRATION (mg/l) OF :-						IC_{50} (mg/l)
		0	0.25	0.5	8	16	32	
271	CPX	2551	-	-	2227	1354	807	16.9
	OFX	3183	-	-	2657	1478	1057	15.2
	NFX	2275	-	-	2275	1789	539	24.3
	NOV	2617	1991	1186	-	-	-	0.46
		0	0.5	1	8	16	32	
273	CPX	3251	-	-	1705	1493	-	11.0
	OFX	2336	-	-	1869	1358	0	17.1
	NFX	1507	-	-	1746	942	603	24.9
	NOV	1582	1392	467	-	-	-	0.82
		0	0.25	4	8	16	32	
274	CPX	2014	-	1738	963	-	-	7.7
	OFX	2922	-	-	1758	927	-	10.9
	NFX	2812	-	-	2531	1546	985	20.0
	NOV	1664	802	-	-	-	-	0.25
		0	0.25	16	32	64	128	
4124	CPX	2649	-	1390	1394	879	-	36.32
	OFX	2864	-	3055	1640	1111	-	44.6
	NFX	2173	-	-	-	2173	611	108.5
	NOV	2270	1135	-	-	-	-	0.25
		0	0.25	0.5	16	32	64	
4133	CPX	4336	-	-	4226	2572	1521	44.3
	OFX	4293	-	-	2377	1916	-	24.0
	NFX	4901	-	-	3910	2781	1839	43.23
	NOV	5577	3495	2919	-	-	-	0.47

STRAIN	DRUG	INTEGRAL AT DRUG CONCENTRATION (mg/l) OF :-						IC_{50} (mg/l)
		0	0.25	8	16	32	64	
4269	CPX	1015	-	808	659	353	-	23.9
	OFX	1403	-	1329	1153	414	-	25.8
	NFX	1875	-	1522	1351	1287	526	46.7
	NOV	1206	119	-	-	-	-	0.71
		0	1	2	16	32	64	
4352	CPX	1146	-	-	1146	326	263	27.2
	OFX	1988	-	-	1288	989	-	31.7
	NFX	2402	-	-	1587	1225	1189	53.3
	NOV	1935	1092	72	-	-	-	1.12
		0	1	2	64	128	256	
4375	CPX	514	-	-	414	183	-	88.3
	OFX	611	-	-	-	464	0	171.7
	NFX	984	-	-	-	688	253	185.7
	NOV	634	-	317	-	-	-	2.0
		0	0.25	0.5	8	16	32	
4495	CPX	5763	-	-	2891	1133	844	16.1
	OFX	4627	-	-	1878	1582	966	6.7
	NFX	4924	-	-	4269	2454	755	15.9
	NOV	2316	1553	585	-	-	-	0.35
		0	1	2	32	64	128	
90-62	CPX	2124	-	-	1384	954	-	55.96
	OFX	2376	-	-	2033	1530	701	90.40
	NFX	1989	-	-	1763	1248	494	85.52
	NOV	5333	4375	0	-	-	-	1.35

STRAIN	DRUG	INTEGRAL AT DRUG CONCENTRATION (mg/l) OF:-							IC50 (mg/l)
		0	2	4	64	128	256	512	
3	CPX	994	-	-	700	716	183	-	180.6
	OFX	1385	-	-	1376	751	578	-	171.3
	NFX	910	-	-	-	910	637	224	368.8
	NOV	410	309	155	-	-	-	-	3.50
		0	0.5	1	32	64	128	256	
4158	CPX	1648	-	-	1596	970	296	-	77.9
	OFX	2218	-	-	1542	984	-	-	56.8
	NFX	1986	-	-	-	1572	1124	746	172.4
	NOV	1987	1475	861	-	-	-	-	0.89
		0	1	2	64	128	256	512	
4374	CPX	3477	-	-	3475	3000	1233	-	219.4
	OFX	6364	-	-	3726	2510	-	-	87.4
	NFX	6212	-	-	4253	3342	3177	2946	334.7
	NOV	5646	3098	2086	-	-	-	-	1.27
		0	0.5	1	8	16	32	64	
4532	CPX	2326	-	-	1575	1274	439	-	18.1
	OFX	2165	-	-	1575	1337	342	-	20.1
	NFX	2001	-	-	1773	1803	1499	847	56.5
	NOV	1606	1569	279	-	-	-	-	0.8
		0	1	2	64	128	256	512	
$C32^R$	CPX	2606	-	-	2092	1766	1528	1109	393.47
	OFX	1592	-	-	1397	1342	985	628	391.53
	NFX	1673	-	-	1629	1414	1218	796	392.49
	NOV	2101	1887	717	-	-	-	-	1.72

STRAIN	DRUG	INTEGRAL AT DRUG CONCENTRATION (mg/l) OF :-							IC_{50} (mg/l)
		0	0.25	0.5	1	16	32	64	
4149^R	CPX	2194	-	-	-	1907	981	-	30.0
	OFX	11467	-	-	-	8559	6373	3691	39.63
	NFX	7892	-	-	-	7606	6127	3847	62.61
	NOV	10954	10069	5289	2093	-	-	-	0.49

STRAIN	DRUG	INTEGRAL AT DRUG CONCENTRATION (mg/l) OF :-								IC_{50} (mg/l)
		0	0.25	0.5	1	8	16	32	64	
C2	CPX	3920	-	-	-	1830	1556	-	-	10.7
	OFX	3242	-	-	-	1836	1556	-	-	14.1
	NFX	3829	-	-	-	-	2345	2067	1667	44.2
	NOV	1823	1684	1555	567	-	-	-	-	0.83
		0	0.25	0.5	1	16	32	64	128	
4125	CPX	8642	-	-	-	7542	3583	-	-	29.02
	OFX	7050	-	-	-	5874	5037	3637	3119	77.8
	NFX	6977	-	-	-	-	6837	3637	673	67.2
	NOV	5095	4058	2599	0	-	-	-	-	0.51
		0	0.5	1	2	4	8	16	32	
4149	CPX	1585	-	-	1571	1386	951	167	258	9.9
	OFX	1359	-	-	-	1032	802	320	-	10.0
	NFX	1227	-	-	-	1023	960	767	418	23.0
	NOV	949	942	649	62	-	-	-	-	1.27
		0	0.5	1	16	32	64	128	256	
4161	CPX	1807	-	-	1815	1577	512	-	-	52.2
	OFX	1739	-	-	1907	495	-	-	-	27.8
	NFX	1705	-	-	-	-	1458	1221	572	200.7
	NOV	2025	1656	87	-	-	-	-	-	0.71

STRAIN	DRUG	INTEGRAL AT DRUG CONCENTRATION (mg/l) OF :-				IC_{50} (mg/l)
		0	2	4	256	
C32	CPX	4740	-	-	4260	>256
	OFX	4795	-	-	2449	>256
	NFX	2998	-	-	1513	>256
	NOV	3560	2918	1492	-	3.59
		0	128	256		
C49	CPX	7974	4906	3933		248.9
	OFX	2395	1727	983		219.1
	NFX	2068	-	1924		>256
	NOV	-	-	-		-
		0	4	8	16	
4131	CPX	334	201	119	-	5.7
	OFX	610	605	263	-	10.6
	NFX	659	-	412	295	13.6
	NOV	-	-	-	-	-

CPX - Ciprofloxacin, OFX - Ofloxacin, NFX - norfloxacin, Nov - Novobiocin.

- reading not taken.

STRAIN	DILUTION	INTEGRAL AT CIPROFLOXACIN CONCENTRATION (mg/l)) OF :-						IC_{50} (mg/l)
		0	32	64	128			
C32	0	6965	-	4312	3209			112.13
	50%	5481	3218	2310	-			48.83
	75%	6027	2605	-	-			28.18
		0	2	4	8	16	32	
90-62	0	2513	1944	1665	-	1987	1647	>32
	50%	2139	-	-	1995	1805	799	27.70
	75%	2305	-	1935	2083	2004	-	-